Search for the Receptor and Target Genes of Sex-Peptide

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von

Elena V. Domanitskaya

aus

Russland

Promotionskomitee

Prof. Dr. Eric Kubli (Vorsitz)

Prof. Dr. Peter Gallant

Prof. Dr. Monica Steinmann-Zwicky

Prof. Dr. Toshiro Aigaki

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Summary

In *Drosophila*, as well as in many other insects, mating significantly changes the physiology and the behaviour of females (Leopold, 1976). Whereas virgin females lay only a few eggs, the egg laying rate is markedly increased in mated females. Furthermore, while virgin females readily accept courting males, they reject courting males after mating (Chen, 1984). Sex-Peptide (SP) is the major player in the stimulation of egg-laying and rejection behaviour in females (the two post-mating responses = PMR). So far several attempts have been made to find a receptor for SP, but unfortunately the identity of the receptor is still unknown. There are two binding sites for SP in the female body: one is localized in the nervous system, the other in the genital tract (Ding et al., 2003; Ottiger et al., 2000). Neuronal binding is dependent on the intact C-terminal part of the peptide and the disulfide bridge. This is the same part of SP that is essential for eliciting the PMR (Schmidt et al., 1993). Genital tract binding is less demanding in terms of peptide sequence. It has been suggested that the binding in the nervous system reflects the presence of a receptor for SP, and that the binding in the genital tract may be due to a carrier protein responsible for the efficient transport of SP into the hemolymph (Kubli, 2003). Based on the fact that cyclic AMP is involved in the response cascade (Bellen and Kiger, 1987), it has been hypothesized that the receptor may be a G-protein coupled receptor (GPCR). To date, two *Drosophila* GPCR encoding genes, *CG13702* and *CG7285*, were proposed for the role of SP receptor based on the structural homology approach (Ding, 2002).

Despite extensive knowledge about the many functions of SP, the receptor and the molecular mechanism of the SP response cascade are mainly unknown. The aim of my PhD thesis was to identify the receptor of SP, and to find components of the SP response cascade in the female.

First, I checked the “homology approach” candidates (*CG13702* and *CG7285*; see above) by RNAi approach. The results clearly showed that none of these genes are involved in the regulation of the PMR, i.e. none of them is the SP receptor. Thus, new attempts to identify the receptor of SP were performed by involvement of microarray and “DUALmembrane system” approaches. Based on the fact that SP neuronal binding sites cannot be detected immediately after eclosion and the binding is fully established 36 h after eclosion, it was assumed that the gene encoding for the binding protein has the same pattern of developmentally regulated transcription. Thus, the microarray approach was applied to identify the neuronal receptor of SP. The expression profiles of mRNA isolated from heads of just eclosed females and from heads of sexually mature females, respectively, were compared with the microarray technique. Gonadotropin-releasing hormone receptor (GRHR) resulted as the most promising candidate gene as it belongs to the GPCRs. Further analysis of *GRHR* transposon insertions showed that

this gene is not involved in the establishment of the PMR, and as a result, it does not encode a SP receptor. Therefore, the “DUALmembrane system” assay of DualSystems Biotech was used to find SP binding proteins by screening two cDNA libraries prepared from the heads of 3 d old females. As a result of the screening, an interaction was observed for SP with the off-track receptor tyrosine kinase (gene: *otk*).

To determine genes which are regulated by SP in the head and in the abdomen to serve the two PMR, I performed a genome-wide expression analysis aimed at identifying all transcripts from the *D. melanogaste*r head and abdomen that exhibit SP-dependent expression patterns. RNA was extracted from females mated with SP^0 males (Liu and Kubli, 2003), and compared with RNA extracted from females mated with control males transferring SP. Hence, a list of SP-induced genes was obtained. To understand the nature and function of the two binding sites in the body of the female (Ding et al., 2003), and to separate the processes that are taking place in the abdomen from those that are taking place in the head, the microarray analysis for RNA extracted from the heads and abdomen, respectively, was performed separately. I found that SP differentially regulates gene expression in the heads and in the abdomen of *Drosophila* females. To determine the contribution of the C-terminal part of SP and the N-terminal part of SP in gene regulation, $SP^{\Delta N}$ mutants were also used in the microarray experiment (Peng et al., 2005a). It was shown that the N-terminal part of SP is not participating in the gene regulation, and that the C-terminal part of SP from aa 8 to 36 is responsible for the regulation of gene expression leading to the two PMR and the activation of the immune response in the abdomen. In the following analysis of the target genes, I concentrated on the genes which are regulated by SP in the abdomen of mated females: *timeless*, and genes coding for the antimicrobial peptides.

Zusammenfassung

Bei Drosophila, wie auch bei vielen anderen Insekten, wird die Physiologie und das Verhalten der Weibchen durch eine Begattung beträchtlich verändert (Leopold, 1976). Während virginelle Weibchen nur ein paar wenige Eier legen, wird die Eilegerate in begatteten Weibchen bedeutend erhöht. Virginelle Weibchen akzeptieren werbende Männchen, nach einer Begattung werden sie jedoch abgewiesen (Chen, 1984). Sex-Peptid (SP) spielt bei der Stimulation der Eiablage und der Zurückweisung der werbenden Männchen (den sogenannten Begattungsreaktionen = BR) die Hauptrolle. Verschiedene Versuche wurden zur Identifizierung des SP Rezeptors unternommen, bisher leider ohne Erfolg. Es gibt im Weibchen zwei Bindungsstellen für SP: eine ist im Nervensystem, die zweite im Genitaltrakt lokalisiert (Ding et al., 2003; Ottiger et al., 2000). Neuronale Bindung von SP ist abhängig von einem intakten C-terminalen Teil und von der Disulfid-Brücke. Dies entspricht demjenigen Teil von SP, der für die Auslösung der BR essentiell ist (Schmidt et al., 1993). Bindung zum Genitaltrakt ist weniger abhängig von der exakten Peptidsequenz. Es wurde daher vorgeschlagen, dass die Bindung im Nervensystem die Anwesenheit eines Rezeptors für SP repräsentiert, während die Bindung im Genitaltrakt auf eine Transportprotein zurückzuführen ist, das für den effizienten Transport des SP in die Hämolymphe zuständig ist (Kubli, 2003). Da cyclisches AMP in die SP Reaktionskaskade involviert ist (Bellen and Kiger, 1987), wurde die Hypothese formuliert, dass der SP Rezeptor ein G-Protein gekoppelter Rezeptor (GPCR) sein könnte. Auf Grund von Ueberlegungen die strukturelle Homologien betreffen, wurden zwei GPCR Gene als mögliche SP Rezeptoren vorgeschlagen: *CG13702* und *CG7285* (Ding, 2002).

Obwohl viele Funktionen des SP gut charakterisiert wurden, sind der Rezeptor und die Reaktionskaskade weitgehend nicht bekannt. Das Ziel meiner Dissertation war es einige mögliche Kandidaten für den SP Rezeptor genauer zu charakterisieren, den SP Rezeptor zu identifizieren und weitere Komponenten der SP Reaktionskaskade im Weibchen zu finden.

Als Erstes wurden die beiden Kandidaten (*CG13702* und *CG7285*) des “strukturellen-Homologie” Ansatzes mittels RNAi untersucht. Die Resultate zeigen klar, dass keines dieser Gene in die Regulation der BR involviert ist, d.h. es handelt sich nicht um SP Rezeptoren. Deshalb wurden neue Versuche unternommen mittels Microarrays und der “DUALmembrane system”-Technik einen SP Rezeptor zu identifizieren. Als Basis diente der Befund, dass die neuronalen Bindungsstellen für SP kurz nach dem Schlüpfen der Weibchen aus der Puppe nicht nachweisbar sind, hingegen 36 Stunden nach dem Schlüpfen voll etabliert sind. Es wurde dabei angenommen, dass die Transkription des für das bindende Protein codierenden Gens diesem Muster folgt. Die Expressions Profile von aus Köpfen soeben geschlüpfter Weibchen und aus Köpfen sexuell reifer Weibchen isolierter mRNAs wurden mittels der Microarray-Technik verglichen. Aus den Resultaten ergab sich, dass der gonadotropin-releasing hormone receptor (GRHR) sich als der beste Kandidat für einen SP Rezeptor erwies, da er zu den GPCRs gehört. Eine Analyse von Transposon-

Insertionen im *GRHR* Gen zeigte aber, dass dieses Gen nicht in die BR involviert ist, d.h. es handelt sich nicht um den SP Rezeptor. Deshalb wurde in der Folge das “DUALmembrane system” vom DualSystems Biotech für ein library screen von SP-bindenden Proteinen eingesetzt. Zwei cDNA libraries wurden aus Köpfen von 3-tägigen Fliegen hergestellt. Als Resultat ergab sich eine Interaktion zwischen SP und der off-track Rezeptor Tyrosin Kinase (Gen: *otk*). Weitere Arbeiten sind aber nötig, um nachzuweisen, dass es sich bei *otk* um den SP Rezeptor handelt.

Um SP-regulierte Gene zu identifizieren die im Kopf und im Abdomen der Weibchen exprimiert sind, wurde eine “Genom-weite” Microarray Analyse der entsprechenden Transkripte durchgeführt. RNAs wurden aus Weibchen isoliert, die mit SP^0 (Liu and Kubli, 2003), bzw. Kontroll-Männchen kopuliert hatten und mittels Microarray Analyse verglichen. Damit wurde eine Liste von SP-regulierten Genen erhalten. Für eine verfeinerte Analyse wurden die RNAs aus Köpfen und Abdomen separat analysiert. Es konnte gezeigt werden, dass SP die Gene im Kopf und Abdomen differentiell reguliert. Um den Beitrag des N-terminalen, bzw. C-terminalen Teils des SP zu bestimmen, wurden transgene Männchen eingesetzt, die ein SP Konstrukt besitzen, denen der N-terminale Teil des SP fehlt (Peng et al., 2005). Es konnte gezeigt werden, dass der N-terminale Teil für die Genregulation keine Rolle spielt. Der C-terminale Teil von SP ist verantwortlich für die Genexpression die zu den BR führt, und auch für die Aktivierung der Immun-Antwort im Abdomen der Weibchen. In der folgenden Analyse der Ziel-Gene habe ich mich auf diejenigen konzentriert, die durch das SP im Abdomen der begatteten Weibchen aktiviert werden: *timeless*, und die Gene die für antimikrobielle Peptide codieren.

1 Introduction

1.1 Products of insect male accessory glands and their functions

The mating behavior and physiology of *Drosophila* females are dramatically modified after copulation. One of the main reasons for this is that *D*. *melanogaster* males try to increase their reproductive success by modulating the postcopulatory state of the female. Male reproductive fitness is promoted by a variety of proteins, which are produced by the accessory glands (Acps), the ejaculatory duct, and the ejaculatory bulb and transferred to the females as components of the seminal fluid (Clark et al., 1995). Acps control a range of processes in the female. For example, Acp36DE is in charge of sperm storage (Neubaum and Wolfner, 1999). Ovulin (Acp26Aa) stimulates release of oocytes in mated females (Heifetz et al., 2005; Herndon and Wolfner, 1995). Sex-Peptide (SP, Acp70A) promotes oogenesis, increases the egg laying rate, (Aigaki et al., 1991; Chen et al., 1988; Soller et al., 1999) and also decreases female receptivity (Chen et al., 1988; Liu and Kubli, 2003). Also, accessory gland secretions, mainly SP, reduce the life span of mated females (Chapman et al., 1995; Wigby and Chapman, 2005). In recent studies using targeted mutagenesis and RNAi, SP was shown to play the major role in eliciting the post mating responses (PMR) (Chapman et al., 2003; Liu and Kubli, 2003). The main emphasis of this work is on SP, thus the peptide is described in detail below.

While Acps, one groupe of the male components modulating the postcopulatory state of females, were intensively studied during last decade, the female side of the post mating biology controlled by Acps such as sperm storage, ovulation, oviposition and fertilization, is still elusive. The first step was performed in two recent genome wide expression studies (Lawniczak and Begun, 2004; McGraw et al., 2004). The identified mating- and Acp-dependent genes in the female contribute to many biological processes including metabolism, immune defense, protein modification, and proteolysis.

1.2 The Sex-Peptide pheromone family

Whereas virgin females lay only a few eggs, the egg laying rate is markedly increased in mated females. Moreover, virgin females readily accept males, and after mating they clearly demonstrate rejection behavior in response to male courtship. The rejection behavior and increased oviposition have been named PMR (Chen, 1984). *D. melanogaster* SP is one of the best characterized peptides affecting female reproductive behavior. SP elicits the PMR in the

female (Chen et al., 1988; Kubli, 2003). It is synthesized in the male accessory glands and during copulation it is transferred into the females. The gene encoding SP has been cloned and sequenced (Styger, 1992). It is a single copy gene containing one intron, and it codes for a 55 aa peptide. The SP gene was cytologically localized to the region 70A on the third chromosome by *in situ* hybridization (Chen et al., 1988). Upon secretion from the main cells of the accessory glands, SP is cleaved in its N-terminal part and releases a signal peptide of 19 aa from the 55 aa precursor (Fig. 1). Mature SP is a linear, unblocked peptide of 36 aa, which contains two cysteines forming a S-S bridge. Furthermore, it contains five hydroxyprolines and, probably, one hydroxyleucine. The biological function of these post translational modifications was not determined, but they are not essential for the PMR (Kubli, 2003).

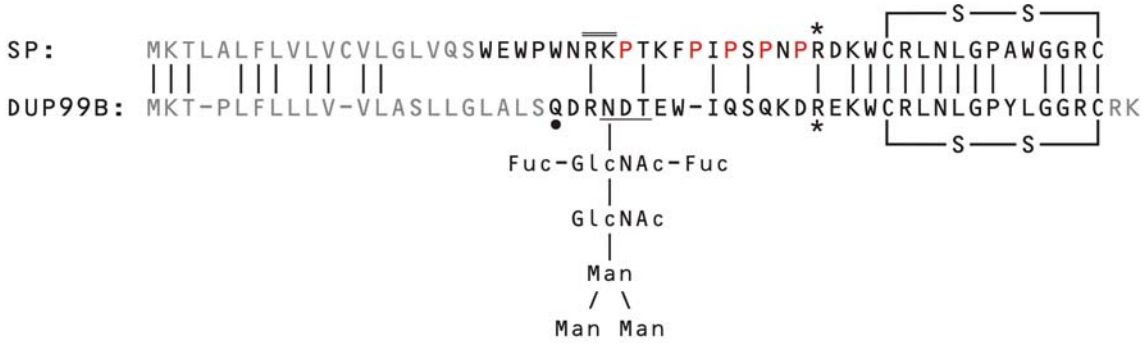

Fig. 1 Amino acid sequences of SP and DUP99B precursors (grey + black) and the mature peptides (black). The mature SP contains 36 aa, the mature DUP99B 31 aa. The SP precursor contains a 19 aa signal peptide (in grey) at its N terminus that is cleaved off during the maturation process. The DUP99B precursor contains N-terminal 21 aa signal peptide and 2 additional aa residues at the C terminus (in grey). The two peptides show high sequence similarity in the N-terminal regions of the signal peptides, and the C-terminal parts of the mature peptides. Identical aa are indicated by vertical bars. Stars, sites of insertion of the introns in the genomic sequences; overlined aa, a trypsin cleavage site, which is important for SP release from the sperm; red P, hydroxyprolines; underlined aa in DUP99B, glycosylation site in the mature DUP99B peptide; filled circle, pyroglutamine (Reproduced from Saudan et al., 2002).

Recently, a second peptide (*Ductus ejaculatorius* peptide cytological region 99B, DUP99B), which is a SP paralogue and also affects egg laying and receptivity (PMR), was isolated and the gene was cloned (Saudan et al., 2002). The *DUP99B* and the *SP* genes contain an intron at exactly the same site. *DUP99B* is expressed in the male ejaculatory duct and in the cardia of both sexes (Rexhepaj et al., 2003). The precursor of the DUP99B peptide contains 54 aa and is cleaved in the N-terminal part (Fig. 1). Furthermore, the last two C-terminal aa of the DUP99B precursor are also cleaved off. Thus, the mature DUP99B peptide is 31 aa long, and has a disulfide bridge in the C-terminal part. It shows high sequence similarity to SP in the C-terminal part, and, thus, the two genes are believed to have arisen by gene duplication (Kubli, 2003). One of the features of DUP99B, not shared by SP, is a standard *Drosophila* glycosyl group in the N-terminal part of the

peptide. DUP99B glycosylation decreases the amount of peptide needed to elicit ovulation (Saudan et al., 2002).

1.3 SP is a multifunctional peptide

After mating, a *D. melanogaster* female lays about 300 eggs in one week (Kubli, 2003). Since ovaries of sexually mature females contain only about 80 - 100 oocytes of the terminal stage 14, after mating, oogenesis must be stimulated. By injection of different fragments of synthetic SP into virgin females it was demonstrated that SP is involved and that it plays a crucial role in the control of oogenesis and oviposition, the so called physiological response (Schmidt et al., 1993a). Also, injection experiments showed that SP is involved in the induction of rejection behavior, the so called neuronal response. The C-terminal end of SP is essential for eliciting these PMR (Fig. 2) (Ding et al., 2003; Schmidt et al., 1993a). Recently, transgenic males lacking SP were produced by targeted mutagenesis by homologous recombination (Rong and Golic, 2000; Rong and Golic, 2001; Rong et al., 2002), the so called SP^0 males (Liu and Kubli, 2003). Since the PMR observed after copulation with SP^0 males were only weakly induced (due to the presence of DUP99B and ovulin), SP is the major player eliciting the PMR (Chapman et al., 2003; Liu and Kubli, 2003).

In an *in vitro* experiment, it was shown that SP induces juvenile hormone (JH) synthesis in *D. melanogaster corpus allatum – corpora cardiaca* complexes (Moshitzky et al., 1996). Experiments with SP fragments showed that the N-terminal part of SP is essential for JH stimulation (Fig. 2) (Fan et al., 2000). SP does also control vitellogenic oocytes development, yolk protein synthesis and uptake (Soller et al., 1997; Soller et al., 1999). Furthermore, the application of the JH analogue methoprene mimics the SP-mediated stimulation of vitellogenic oocytes progression in sexually mature virgin females (Soller et al., 1999). JH seems to be a downstream component in the SP response cascade that stimulates vitellogenic oocytes progression and inhibits apoptosis. But the JH analogue cannot induce the two PMR (Soller et al., 1999).

The N terminus of SP is also important for binding to sperm, the gradual cleavage and release of the C-terminal part of SP from the sperm tail (Fig. 2) (Peng et al., 2005a). The C-terminal part of SP is very likely transported via hemolymph to the CNS where it elicits the PMR. Binding to sperm may protect the peptide from degradation by proteases in the hemolymph and, thus, prolong its half-life (Peng et al., 2005a).

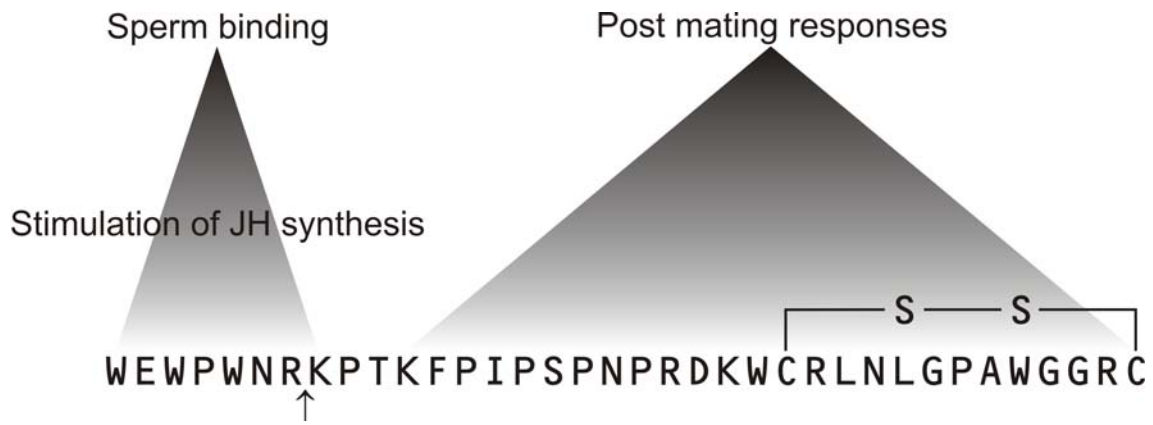

Fig. 2 Compilation of known functions of SP. Results from *in vitro* and *in vivo* experiments. Shaded are regions of functional importance. Arrow designates a trypsin cleavage site, which is important for SP release from the sperm (Reproduced from Saudan et al., 2002; Peng et al., 2005).

1.4 SP receptor and SP response cascade

Although SP and the PMR have been studied for almost 20 years, the gene coding for the SP receptor and the SP response cascade are still unknown. Identification of the gene coding for the SP receptor is an important step in our understanding of SP action. Here, I will summarize what is known about the SP receptor and its mode of action.

1.4.1 One type of molecular receptor for two PMR

By injecting SP and fragments thereof into the abdomen of virgin females it has been found that the absence of the first 10 N-terminal amino acids does not affect the induction of the PMR, whereas all fragments either with removed C-terminal end of SP, or with a destroyed disulfide bridge, are inactive (Ding et al., 2003; Schmidt et al., 1993b). Neither of the post translational modifications of SP is essential to elicit the PMR (Saudan et al., 2002; Schmidt et al., 1993b). SP fragments elicit either both or none of the PMR (Schmidt et al., 1993b). Also ectopic expression of membrane-bound SP in different tissues, led either to both responses or none (Nakayama et al., 1997). Dose response curves show that a critical concentration of about 0.6 pmol/female is needed to elicit oviposition or reduction of receptivity (Moshitzky et al., 1996; Schmidt et al., 1993a). These findings indicate that there might be only one type of molecular receptor on the top of the SP response cascade leading to the two PMR (Fig. 3).

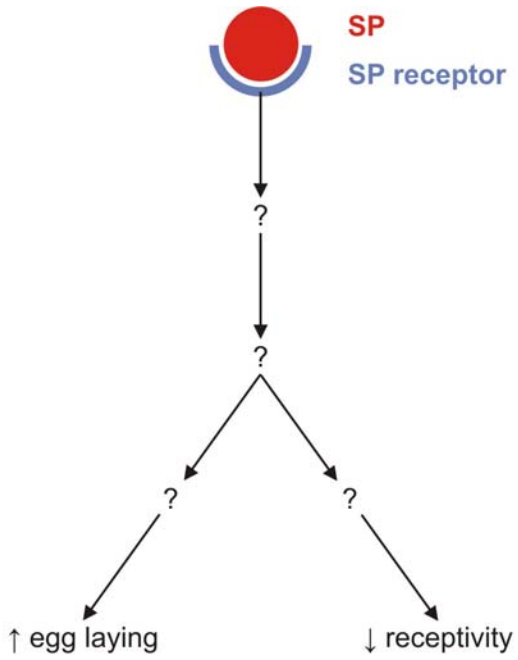

Fig. 3 Hypothetical model of SP action. There might be one molecular receptor on the top of the SP response cascade leading to the two PMR.

1.4.2 SP target tissues

The localization of SP-binding proteins at the tissue level was investigated by incubating cryostat tissue sections of females with radiolabeled ^{125}I-YsSP (a tyrosine residue Y was added at the N-terminus of the synthetic peptide for iodination purposes) (Ottiger et al., 2000). Labeling was found in the central nervous system (subesophageal ganglion, the cervical connective, and in discrete parts of the thoracal-abdominal ganglion), the peripheral nervous system (the proximal parts of leg nerves, the wing and haltere nerves, and the antennal-, labial-, and accessory pharyngeal nerves), and in the genital tract (uterus and oviduct) (Fig. 4). A similar binding pattern in the CNS and PNS was found in male sections. Moreover, different fragments of SP bind differentially to the target sites (Ding et al., 2003). Neuronal binding is dependent on the intact C-terminal part of the peptide and the S-S disulfide bridge. However, genital tract binding is less demanding in terms of peptide sequence.

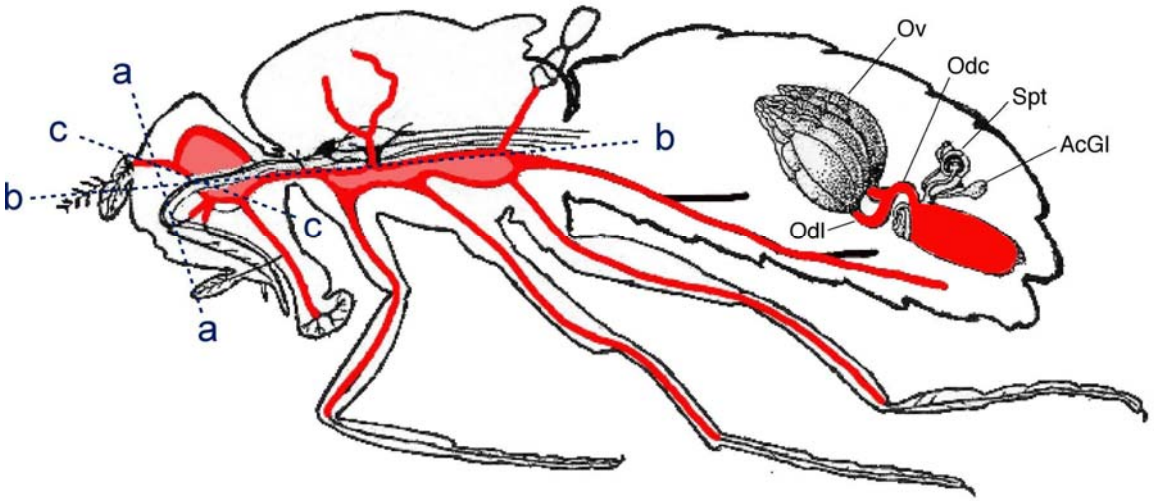

Fig. 4 Schematic overview of SP binding sites in the nervous system and genital tract. Dark red, strong labeling; light red, weak labeling; black, no labeling. The binding to the nervous system very likely represents binding to a receptor, whereas binding to the genital tract reveals the presence of a carrier protein for SP. AcGl, female accessory gland; Odc, common oviduct; Odl, lateral oviduct; Ov, ovary; Spt, spermaticae (Reproduced from Ding et al., 2003; Ottiger et al., 2000).

Affinity blots of cytosolic and membrane extracts prepared from female heads and abdomen, respectively, were probed with the fusion protein AP-SP. Two proteins, that differ in their molecular properties, were detected in the membrane fractions (Ding et al., 2003). These data allow the conclusion that SP has two different (transmembrane or membrane-associated) receptors: one expressed in the CNS, and the other in the genital tract. Moreover, it was found that the target sites in the nervous system and in the genital tract appear at different developmental stages. In the genital tract the binding sites appear already in the last pupal stage, the neuronal binding sites cannot be detected until immediately after eclosion. Thirty six hours after eclosion, labeling is at the same level of intensity as in 5 d old, sexually mature females (Ottiger et al., 2000). It has been suggested that the binding in the nervous system reflects a receptor for SP at the top of a signaling cascade leading to the two post-mating responses, whereas the binding in the genital tract might reflect a carrier protein responsible for the efficient transport of SP into the hemolymph (Kubli, 2003). SP is believed to be transferred into the hemolymph where it reaches the CNS. This view is supported by ectopic, constitutive expression of SP in the fat body of females secreted into the hemolymph leading to a continuous induction of the PMR (Nakayama et al., 1997).

1.4.3 Involvement of cyclic AMP in the SP response cascade

Females of the *D*. *melanogaster* mutant *dunce* do not respond to SP and mate at least twice as often as females of wt stocks (Harshman et al., 1999). The gene *dunce* encodes a cAMP-specific phosphodiesterase II, and in the *dunce* mutants the level of cAMP is elevated (Byers et al., 1981). Among other effects are learning abnormalities, memory deficiency and female sterility (Byers et al., 1981). Based on these facts, it was concluded that cAMP may be involved in the SP response cascade directly or indirectly (Fleischmann, 1997). Since the *dunce* gene is mainly expressed in the mushroom bodies (Nighorn et al., 1991), studies with chemical ablation of mushroom bodies and its effect on the SP-induced PMR were carried out (Fleischmann et al., 2001). The ablation of the mushroom bodies resulted in an increased background egg laying rate in virgin females, but did not affect the PMR induced by SP. Thus, mushroom bodies are not essential for eliciting the SP response cascade. *Rutabaga*, another memory and learning mutant, does also not respond to SP (Fleischmann, 1997). The gene *rutabaga* encodes a Ca_2^+/calmodulin (CaM)-responsive cyclase, and the level of cAMP is decreased in the mutant flies (Fleischmann, 1997).

Since cyclic AMP is involved in the response cascade (Bellen and Kiger, 1987), the receptor of SP might be a G-protein coupled receptor (GPCR).

1.4.4 Two candidate SP receptors suggested by a structural homology approach

Since a *D*. *melanogaster* cDNA library expressed in COS cells with an alkaline phosphatase – SP fusion protein was screened without any positive results (Ding, 2002), another approach was applied to search for the SP receptor. Based on the assumption that the SP receptor might be a GPCR, and that appropriate conformations of ligand and receptor are needed for successful and correct ligand – receptor interactions, a structural homology approach was applied to find the SP receptor. Since the S-S bridge is essential for SP binding to the CNS in *D*. *melanogaster* (Ding et al., 2003), peptides interacting with GPCRs and which had a similar S-S structure were searched in vertebrates (Ding, 2002). The somatostatin family has structural and biochemical similarities with SP (Janecka et al., 2001). As in SP, the S-S disulfide bond of the somatostatin family is necessary for binding to its receptors. Although somatostatin-like receptors were identified in the fly genome, there was no ligand found for somatostatin-like receptors in *D*. *melanogaster*. Hence, SP was assumed to be the somatostatin-like counterpart in the fly. Forty-four neuropeptide GPCRs were described in the *Drosophila* genome (Hewes and Taghert, 2001), and, as mentioned above, the SP receptor may belong to the GPCR family. Among the 44 *Drosophila* neuropeptide receptors, two putative somatostatin receptors AICR2 (gene: *AICR2* or *CG13702*)

and Star1 (gene: *star1* or *CG7285*) were identified (Ding, 2002). Unfortunately, there is nothing known about the expression pattern, the function and ligands of these receptors. Thus, to confirm that one of these genes may indeed encode the SP receptor, additional experiments are necessary.

1.5 Aim of the thesis

The identification of a gene coding for the SP receptor will be a crucial step forward in the characterization of the SP response cascade. On the other hand, the identification of the genes that are regulated by SP in the head and in the abdomen and which serve the two PMR, might help us to find out the mode of SP action and the SP receptor. Hence, the goal of my thesis was to identify the receptor for SP, and to find components of the SP response cascade. Since, there are two binding sites for SP in the female body, CNS and genital tract, which differ in their molecular properties, I wanted to identify genes regulated by SP in the head and in the abdomen that may serve the two post-mating responses. Another important question to be answered was the contribution of the C- and N-terminal part of SP in these post-mating responses is.

2 Materials and methods

2.1 Flies

All flies were kept on standard cornmeal/yeast/agar medium at 25°C.

2.1.1 SP mutants

The following mutant *Drosophila* strains were used (Liu and Kubli, 2003): SP^0 (0325/TM3, *Sb*, *ry*, mutant stock); $Control^{SP}$ (0416/TM3, *Sb*, *ry*, rescue stock for SP^0 with wt SP gene); $SP^{\Delta 2-7}$ (*w*; Cd/Cd; Δ130/TM3, *Sb*, *ry*, SP deficiency stock with N-terminally deleted amino acids from 2 to 7, on the second chromosome); $Control^{\Delta 2-7}$ (*w*; C0/CyO; Δ130/TM3, *Sb*, *ry*, control stock for $SP^{\Delta 2-7}$ with wt rescued SP); Δ130/TM3 (SP deficiency stock).

2.1.2 Stocks for candidate SP receptors

RNAi transgenic flies

The genetic background of all lines is w^{1118}. An inverted unique sequence of a gene (*star1* or *AICR2*) was cloned as antisense/sense into a modified pUAS with 10XUAS. The "Ver Primer" and "Second Cloning Primer Seq" are two primers used to PCR the target sequence for the hairpin (Table 1). PCR on genomic DNA was done to check for the presence of the hairpin from the "Ver Primer" and a constant primer. The constant primer was designed from the hsp70 promoter of the UAS (hsp7: AAGCAAAGTGAACACGTCGCTAAGC). The PCR was done for 38 cycles at 59°C.

***Off-track* (*otk*) mutants**

EP2017 flies carry a P element insertional mutation (designated EP2017) 30 bp upstream of the 5' end of the *otk* cDNA. The otk^3 mutation is lethal and carries a 3 kb deletion that extends downstream of the EP2017 element, disrupting *otk* but not upstream genes. The otk^3 lesion represents a complete loss-of-function allele.

Name of the gene	Construct ID	Verification PCR length	Chromo-some	Ver Primer Seq	Second Cloning Primer Seq
star1 (*CG7285*)	7C11	453 bp	2	CGCGAATTCCTG TGGTGCTCTACG GATTTGTGTG	CGCTCTAGATG CGTCGGTCGAT TCCTGTG
AICR2 (*CG13702*)	7B11	543 bp	2	CGCGAATTCGGC GAGGAGGAGGA GGAGGAG	CGCTCTAGAGA CGAGGTGAAG GAGGTGATGG A

Table 1 Information about RNAi transgenic flies (Barry Dickson, IMBA, Vienna).

2.1.3 *osk* flies (for proteomics approach)

To generate *osk* flies (lacking the germline), homozygous osk^{1}/osk^{3} females were crossed to wt males. The offspring of this cross lacks a germline, and thus "contamination" with yolk proteins is avoided: females cannot produce eggs (and males cannot generate sperm). It was shown that *osk* females respond to SP (Ding et al., 2003).

2.1.4 Reporter lines for AMP genes

Mtk-GFP reporter line

The Mtk-GFP stock is a transgenic line generated in the w^{-} background and carrying a reporter gene containing 1.5 kb of *Metchnikowin* upstream sequences fused to the green fluorescent protein (GFP) cDNA. Furthermore, 790 bp of 3' flanking region of the *Drosomycin* gene were used to signal transcription termination and polyadenylation (Levashina et al., 1998). A transgenic line with an insertion on the third chromosome was used in this study (Levashina et al., 1998).

Drc2.5-*lacZ*-2

The Drc2.5-*lacZ*-2 stock is a transgenic line generated in the ry^{506}CSA background and carrying a reporter transposon consisting of a 2.5-kb *Drosocn* promoter fragment fused in the leader sequence to the bacterial *lacZ* gene followed by a *Drosocin* downstream sequence (Charlet et al., 1996). Two independent transgenic lines (transposon inserted either on the second or on the X chromosome) were used in this study.

2.2 Bioassays

2.2.1 Receptivity test

One female (4-5 d old) was placed together with 2-3 males (4-5 d old) in an empty vial. The number of copulations of 20-40 females within 60 min was recorded. Receptivity was expressed as percentage of the copulated females. Each experiment was repeated 3 times.

2.2.2 Egg laying rate

Five d old virgin females of various genotypes were mated with 5 d old males of various genotypes. Eggs laid by individual females were counted in a 24 h period. Between 15 and 20 females were included in each experiment. Each experiment was repeated 3 times.

2.3 Microarray experiments

2.3.1 Preparation of biotinylated cRNA

Total RNA was isolated from 100 heads or 30 abdomen of females with the reagent TRIzol (GIBCO BRL). Freshly dissected or stored (-70°C) heads or abdomen, respectively, were homogenized in 50 μl of TRIzol, then filled up to 800 μl with TRIzol, and 10 μg of glycogen (Roche) added. The mixture was incubated at RT during 5 min and afterwards 160 μl of chloroform was added, vortexed for 15 sec and incubated during 2 - 3 min at RT. The sample was centrifuged in a Benchtop centrifuge at maximum speed during 15 min at 4°C. The aqueous phase was taken out, 1 vol of isopropanol was added and the sample was incubated for at least 1 h up to overnight at -20°C. Then it was centrifuged during 10 min at 4°C, washed twice with 75% EtOH, 7000x g, 5 min, at 4°C. After precipitation and washing, the RNA was dissolved in 20 μl DEPC-treated water (Ambion) and spectrophotometrically quantified by using a UVIKON 722 LC, BIO-TEK KONTRON spectrophotometer. The $OD_{260/280}$ of total RNA was measured, and the RNA quality was checked on the Agilent 2100 Bioanalyser of the FGCZ (Functional Genomics Center Zurich, UNI/ETH Zurich). cDNA was synthesized on total RNA as a template using the SuperScript Choice System for cDNA synthesis (Life Technologies) with a T_7-$(T)_{24}$ DNA primer: 5'- GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG$(dT)_{24}$ - 3'. For first strand cDNA synthesis, a typical 40 μl reaction contained 25 μg RNA, 100 pmol T_7–$(T)_{24}$ primer, 10 μM of each dNTP, and 500 units of reverse transcriptase (SuperScript (SSR II) RT; Invitrogen). The reaction

was incubated for 1 h at 42°C. Second-strand cDNA synthesis was carried out at 16°C for 2 h in a total volume of 150 μl by using 10 units *E. coli* DNA ligase, 40 units *E. coli* DNA polymerase I, and 2 units RNase H in the presence of 30 mM of each dNTP. After second-strand cDNA synthesis, 10 units of T4 DNA polymerase were added, and the samples were incubated at 16°C for 5 min. After that, 10 μl of 0.5 M EDTA was added and double-stranded cDNA was cleaned up with the GeneChip Cleanup Module (Affymetrix). After cleaning, the samples were stored at -20°C. Biotinylated antisense cRNA was synthesized from the double-stranded DNA template using the Enzo BioArray High Yield RNA Transcript labeling Kit (T7) (Enzo). Forty μl reaction volume contained approximately 1 μl of transcribable cDNA template. The reaction mixture was incubated at 37°C for 4.5 h with gentle mixing every 30 – 45 min. Thereafter, the samples were cleaned with the GeneChip Sample Cleanup Module (Affymetrix) and quantified on the agilent 2100 Bioanalyzer. Fifteen μg of the biotinylated antisense cRNA were added to the fragmentation buffer (2 μl 5x fragmentation buffer for every 8 μl cRNA) (supplied with GeneChip Sample Cleanup Module, Affymetrix), and taken up in 20 μl DEPC-treated water. Thereafter, biotinylated antisense cRNA was fragmented by heating the samples to 95°C for 35 min and then put on ice.

2.3.2 High-Density Oligonucleotide Arrays

In this study, a custom-designed Affymetrix GeneChip Drosophila Genome Array (P/N 900335, Affymetrix, Santa Clara, CA) was used. The genes represented on the array correspond to more than 13500 mRNA transcripts from known genes and predicted ORFs. This includes genes for which confirming EST or full-length cDNA evidence is available (over 8000 genes have at least 1 EST/cDNA match) and genes currently based solely upon prediction algorithms or homology. Each gene is represented on the array by a set of 25mer oligonucleotide probes matching the gene sequence. To control the specificity of hybridization, the same probes are synthesized with a single nucleotide mismatch in a central position. Each gene is represented by 14 probe arrays used to measure the transcript level of the sequences. The difference between the perfect match (PM) hybridization signal and the mismatch (MM) signal is proportional to the abundance of a given transcript. The oligonucleotide probe selection corresponding to each *Drosophila* gene and the array fabrication was performed by Affymetrix.

2.3.3 Hybridization

Gene chips were prehybridized with 200 μl hybridization buffer (1x MES, pH 6.5-6.7; 1 M NaCl; 0.01% Triton-X-100; 0.04 M EDTA) for at least 10 min at 45°C with 60 rpm rotation (GeneChip

hybridization oven). Hybridization was done in a final volume of 300 μl hybridization buffer, containing 15 μg fragmented biotinylated cRNA, 30 μg herring sperm DNA, 150 μg acetylated BSA (Sigma), 15 pM B2 control oligo (Affymetrix) and Eukaryotic Hybridization control mix (Affymetrix). The samples were heated to 99°C for 5 min; afterwards the samples were transferred to a 45°C heat block for 5 minutes and briefly spun down. Hybridizations were carried out for 16 h at 45°C with 60 rpm rotation in the hybridization oven. After hybridization, the solutions were removed, and the probe arrays were filled with 250 μl of non-stringent wash buffer (6x SSPE, 0.01% Tween-20) and washed on a Fluidics station of the FGCZ (Affymetrix). The hybridized and washed probe arrays were next stained with 600 μl detection solution. Detection solution is a 1x stain buffer (1x MES, 1.8 M NaCl, 0.01% Tween-20) containing streptavidin-phycoerythrin conjugate (Streptavidin R-phycoerythrin, 1 mg/ml; Molecular Probes) and 1.2 mg acetylated BSA (Sigma). I have used a signal amplification protocol (fluidics protocol EukGE-WS2, Affymetrix) by using antibody amplification. 600 μl stain buffer contains 60 μg goat IgG (10 mg/ml; Sigma), 1.8 μg biotinylated antibody (0.5 mg/ml; Reactolab S.A.), and 1.2 mg acetylated BSA (Sigma).

2.3.4 Scanning and data analysis

Probe arrays were scanned with a commercial argon-ion laser scanner (Agilent GeneArray Scanner). Pixel intensities were measured, and expression signals were analyzed with the Microarray Suite 5.0 software. Detailed data analysis was carried out using the GeneSpring 5.0 program (Silicon Genetics).

Data analysis for microarray experiment based on the developmental regulation of the SP receptor

Three replicates consisting of heads of 100 females just after eclosion each (condition 1) and three replicates consisting of heads of 100 3 d old females each (condition 2) were carried out. For the analysis of expression profiling, condition 1 was assumed as the control, i.e. the up-regulated genes were the genes of interest. All signal intensities of one sample (chip) were divided by the respective median, resulting in a median per chip normalization. Subsequently, a per gene normalization to the control values was carried out in the following way. All per chip normalized values belonging to one gene were divided by the mean of the respective control values. For the analysis of expression profiling, three filter operations were combined. The first filtering was based on the Affymetrix MAS 5.0 detection calls. Only genes for which I found at least 2 “P” (Present) flags out of 3 samples for condition 2 were accepted. The reason for this

was the idea, that the genes of interest should be up-regulated in condition 2. The condition 1 could contain “M” (Marginal) and “A” (Absent) flags, since the genes of interest are either not expressed or expressed at low level in females just after eclosion (Ding et al., 2003). As the second filtering step, I applied a Weleh t-test. Only genes showing a p-value of 0.05 or smaller were accepted. Finally, I selected genes for further analysis that showed at least two fold changes in the expression from condition 1 to condition 2.

Data analysis for genome-wide analysis of SP response

Three replicates consisting of RNA extracted form the heads of 100 wt females 4 h after copulation with SP^0 males each (condition 1), and three replicates consisting of RNA extracted from the heads of 100 wt females 4 h after copulation with $Control^{SP}$ males each (condition 2) were carried out. All signal intensities of one sample (chip) were divided by the respective median, resulting in a median per chip normalization. Subsequently, per gene normalization to the control values was carried to the specific sample divided by the respective mean. Afterwards, the following filtering steps were applied: 3 PM (at least in 3 samples out of 6, a gene should have a Present or Marginal flag), 2-fold change (I assumed genes to be significantly up- or down-regulated if they demonstrate an at least 2-fold change in their expression profiles), 1-way ANOVA (Weleh t-test, p=0.05).
The same steps were carried out for the profiling of gene expression in the abdomen of females mated with SP^0 males in comparison with females mated with $Control^{SP}$ males, as well as in the heads and abdomen of females mated with $SP^{\Delta 2-7}$ males in comparison with females mated with $Control^{\Delta 2-7}$ male.

2.4 QRT-PCR

Total RNA was prepared as described in 2.3.1 using TRIzol, followed by DNase treatment to control for amplification of background genomic DNA in the RNA samples (Ambion, *DNA-free*, Cat. No. 1906). Accurate quantification of total RNA was performed. Total RNA was reverse transcribed by using the Qiagen reverse transcription system (Qiagen, Cat. No. 205111). No-RT reactions (no reverse transcriptase) were used to control for amplification of background genomic DNA in the RNA samples. Each QRT-PCR was performed in triplicates using SYBR Green PCR Core Reagents (Applied Biosystems, Foster City, CA). *Rpl32* (60S ribosomal protein L32), *tub* (tubulin), *act* (actin) and *TBP* (TATA binding protein) were used as reference control genes. The QRT-PCR data were analyzed using the comparative CT method (Livak and Schmittgen, 2001). Briefly, the difference in cycle times, ΔCT, was determined as the difference between the tested

gene and the reference housekeeping gene, GAPDH. I then obtained ΔΔCT by finding the difference between treatments. The fold change was calculated as $FC=2^{-\Delta\Delta CT}$. To determine confidence intervals, I used replicate measurements and experiments to model the variability in ΔΔCT with a normal distribution. I then converted the intervals to the fold-change scale.

2.5 β-galactosidase staining of the female genital tract

Female genital tracts were dissected and collected in PBS. The tissues were fixed with 4% paraformaldehyde (PFA) in PBS for about 20 min at room temperature. The PFA solution was removed and the genitalia were rinsed twice in PBS. The tissue were then incubated in 1 ml X-Gal solution (500 μl buffer B, 5 μl 0.3 M ferricyanide, 5 μl 0.3 M ferrocyanide, 5 μl 10% Triton-X-100, 5 μl 10% X-Gal solution in dimethylformamide at 37°C, in the dark, over night. After removing the X-Gal solution, the tissues were washed with PBS and mounted in 60% glycerol in PBS. Buffer B was made of 10 mM sodium phosphate buffer, pH 7.0; 1 mM $MgCl_2$; 150 mM NaCl; ddH_2O.

2.6 Proteomics

2.6.1 Cell culture and production of AP-SP_{8-36} fusion protein

Cos-7 cells were used for transient transfection by electroporation. Transient transfection was done with the APtag-4 (Flanagan and Cheng, 2000; Flanagan et al., 2000) or AP-SP_{8-36} (Ding et al., 2003) vectors. These vectors contain a simian virus 40 (SV40) origin, thus they can replicate in cell lines that express the SV40 large T antigen, such as COS cells or 293T cells (American Type Culture Collection, Manassas, VA) (Ding et al., 2003; Flanagan and Cheng, 2000; Flanagan et al., 2000). The APtag4 vector was used to generate AP fusion proteins for the control experiments. Cells were grown in Dulbecco's modified eagle's medium (DMEM) with 10% fetal bovine serum at 37°C under 5% CO_2 in 100 mm tissue plates until the cells reached 50-60% confluence.

Before trypsinisation cells were washed with PBS. Trypsinized cells were resuspended with medium to inactivate trypsin in the serum, and harvested by centrifugation during 5 min at 1000 rpm. The supernatant was sucked off (on ice) followed by washing of the cells with PBS and centrifugation during 5 min at 1000 rpm. The PBS was discarded and the cells were resuspended in ice-cold PBS containing 5-15 μg vector DNA. 800 μl aliquots of the cell suspension were

transferred into electroporation cuvettes (BTX Disposable cuvette P/N 640, 4 mm gap). Cuvettes were placed in a holder in the electroporation apparatus at room temperature and shocked with 300 V, 500 μF. Transfected cells were 28-fold diluted in non-selective complete medium and sieved on the 100 mm tissue plates. After 70 h, the supernatant of the cell culture was collected by centrifugation at the maximum speed in a Benchtop centrifuge and buffered with 10 mM HEPES, pH 7.0 (Merck). 0.05% NaN_3 was added to prevent microbial growth.

2.6.2 Quantitative measurement of activity of APtag fusion proteins

Since each fusion protein contains one APtag, the concentration of the fusion protein can be estimated from the AP activity. AP activity is measured by adding the substrate *p*-nitrophenyl phosphate, which is converted into a yellow product that can be quantified (Flanagan et al., 2000). One μl of supernatant was diluted into 500 μl HBAH buffer (0.5 mg/ml BSA (Invitrogen); 0.1% NaN_3; 20 mM HEPES, pH 7.0), and heated at 65°C for 10 min to inactivate the endogenous AP activity. The tubes were spun down in a microcentrifuge (Eppendorf) at maximum speed for 5 min and the supernatant was collected. Then, 500 μl 2x AP buffer (24 mM *p*-nitrophenyl phosphate (Sigma), in 2 M diethanolamine containing 1 mM $MgCl_2$, 20 nM L-homoarginine (Sigma)) were added to make the total enzymatic reaction 1 ml. After incubation for 1 h at room temperature, OD values at 405 nm were measured in a 1 ml cuvette in a spectrophotometer (UVIKON 722 LC, BIO-TEK KONTRON). To convert the OD units per hour into pM of AP protein, the OD units were divided by a conversion factor of 36 (Flanagan et al., 2000).

2.6.3 Protein extraction

Different parts of the body (heads or abdomen) were freshly collected (cut off on ice) and immediately homogenized in homogenization buffer (50 nM Tris-HCl, pH 7.6, 150 mM NaCl, 1 mM PMSF, proteinase inhibitor cocktail (Roche)), and centrifuged at 10'000 x g at 4°C for 1 hr to remove soluble material. The supernatant (cytosolic extract) was collected and used as a control for further experiments. The resulting pellet (crude membrane extract) was resuspended in homogenization buffer containing 1% Triton-X-100 and incubated on ice for 20 - 30 min to solubilize the membrane extract. Afterwards the sample was centrifuged at low speed (1000x g) at 4°C for 20 - 30 min to clarify the membrane extract from debris or particles. The supernatant containing the membrane protein fraction was collected, added loading buffer and immediately loaded onto a Native PAGE (Ding et al., 2003).

2.6.4 Preparative Native PAGE

Six % polyacrylamide resolving (6 cm) and 4% stacking (3 cm) gels were cast in the gel tube of the Mini-PROTEAN II cell (BIO-RAD). Non-denaturing PAGE was carried out in a discontinuous electrophoresis buffer system. The 2x buffer used to prepare the resolving gel, was 0.75 M Tris-HCl, pH 8.8, and the 2x buffer used in the stacking gel was 0.25 M Tris-HCl, pH 6.8. I used 30% Acrylamide/Bis stock solution (37.5 : 1), because in Native PAGE, gels with large pores are preferred to allow running samples at relatively high migration rates. Once the gels were polymerised, the apparatus was set up with a native electrode running buffer (25 mM Tris, 0.192 M Glycine, pH 8.3). A sample for loading on the gel was prepared by adding to the membrane or cytosolic extracts a Native PAGE sample buffer (62.5 mM Tris-HCl, pH 6.8; 10% Glycerol, 0.025% Bromophenol Blue) (1:1). The same sample was loaded onto two neighboring wells. Fifty V were applied to run samples through the stacking gel and 100 V were used to run them through the resolving gel. Afterwards the gel was cut according to the wells and used in further experiments: one of them for affinity blotting and the other for Coomassie Blue staining and further LCQ-MS/MS analysis.

2.6.5 Blotting

After running the gel, the proteins were transferred onto NC membranes (BIO-RAD) in the semi-dry transfer buffer (39 mM glycine, 48 mM Tris-base, 20% methanol). The transfer was carried out using the semi-dry method (LKB, NOVABLOT) at 15 V for 3 h.

2.6.6 Incubation with AP-SP_{8-36} and staining

After blocking of the membranes in 5% non-fat milk overnight at 4°C with gentle shaking, the membranes were incubated with an AP-SP_{8-36} probe or APtag-4 (as a control) in the blocking solution at room temperature on the shaker for about 1.5 hr. Then the membranes were washed with two changes of Tris-HCl buffer (50 nM Tris-HCl, pH 7.6, 150 mM NaCl) and incubated with NBT/BCIP substrates in the alkaline phosphatase buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-HCl, pH 9.5) in the dark (!) to develop the color reaction. Positive staining appeared in about 40 min, false positives tended to appear only several hours later.

2.7 The DUALmembrane system (MbYTH, see also Appendix)

2.7.1 Construction of prey construct

To generate a prey construct the *pNDB1* vector containing Cub fused to the LexA-VP16 transcription factor was used. A DNA fragment encoding a putative transmembrane receptor can be fused to the LexA-VP16-Cub in such a manner that the product of this construct exposes the C-terminus of the transmembrane receptor outside of the cell. Since SP is a small extracellular peptide, it was fused to the transmembrane anchor Sec12. A cDNA coding for the Sec12 transmembrane glycoprotein from aa 339 to aa 470 was amplified by PCR from the following primers: f-pst-sec12 (AAACTGCAGACCGCTTAACTACGCCAACTACACC, Tm 74°C) and r-nco-sec12 (TGATGCCATGGAGCATCATCTATTTCTCGAAAAGTTGC, Tm 72°C), and cloned into the multiple cloning site of the *Pst*I- and *Nco*I- digested *pNDB1* vector. A DNA fragment encoding the mature SP was amplified by PCR from total RNA extracted from the accessory gland of 5 d old wt males from the following designed primers: f-nco-sp (ACATGCCATGGTGGGAATGGCCGTGGAATAGGAAG, Tm 74°C) and r-sacii-sp (TCCCCGCGGTTAACATCTTCCACCCAGGCGG, Tm 72°C), and cloned into the multiple cloning site of the *Nco*I- and *Sac*II- digested *pNDB1* vector. The resulting vector (*pNDB1-Sec12-SP*) contained the LexA-VP16-Cub-Membrane anchor-SP construct. Plasmid sequences and detailed construction schemes of all constructs used in this study are available upon request from Dualsystems Biotech (www.dualsystems.com).

2.7.2 Construction of the NubG-fused random *Drosophila* female head cDNA library

To generate the NubG-x cDNA library, total RNA was extracted from the heads of 3 d old females (for the RNA extraction method see 2.3.1) for the first strand cDNA synthesis from oligo dT primers (ATTCTAGAGGCCGAGGCGGCCGACATG$(T)_{30}$VN). The library was introduced in the *pDSL-NubG* vector (NubG-x orientation, where x is an insert of cDNA) at the *Sfi*I digestion site and had a complexity of $3.0x10^7$ clones. The subsequent analysis showed that the percentage of recombinants is 100% and that the average insert site is 1.5 kb with a size range from 0.4 to 2.5 kb.

To generate the x-NubG cDNA library, total RNA was extracted from the heads of 3 d old females (for the RNA extraction method see 2.3.1) followed by the poly-A^+ RNA extraction (Qiagen, Oligotex mRNA Mini Kit, Cat. Nr. 70022). Poly-A^+ RNA was used for the first strand cDNA

synthesis from random hexamer primers (ATTCTAGAGGCCGAGGCGGCCGACATGNNNNNN). The library was introduced into the *pDL2xN-SUC-NubG* / *pDL2xN-STE-NubG* vector (x-NubG orientation, where x is an insert of cDNA) at the *Sfi*I digestion site and had a complexity of $2.6x10^6$ clones. The subsequent analysis showed that the percentage of recombinants is 80% and that the average insert site is 1.8 kb with a size range from 1.2 to 2.5 kb.

Requests for the NubG-x and x-NubG *Drosophila* female head cDNA libraries should be directed to Dualsystem Biotech (www.dualsystems.com).

2.7.3 SP split-ubiquitin membrane yeast two-hybrid screen

In the library screen, the reporter strain expressing the bait is transformed with both female head cDNA libraries (NubG-x and x-NubG). Two independent transformations were carried out for both libraries. Primary selection was carried out on selective medium lacking the amino acid histidine, secondary selection was carried out using a semi-quantitative color assay for β-galactosidase.

3 Results

3.1 Search for the receptor of SP

3.1.1 AICR2 and Star1 are candidate SP receptors based on a structural homology approach

Based on the fact that cyclic AMP is involved in the SP response cascade (Bellen and Kiger, 1987; Chapman et al., 1996) it has been hypothesized that the SP receptor may be a G-protein coupled receptor (GPCR). About 50 GPCRs have been identified in the *D. melanogaster* genome based on sequence homology to the corresponding vertebrate receptors (Hewes and Taghert, 2001). Since the ligands are peptides, their aa sequence may have diverged beyond recognition. However, secondary structure might be conserved. Vertebrate somatostatin contains a S-S bridge at the C-terminal end as SP, i.e. it very likely possesses a similar secondary structure, but no further aa homologies. Since somatostatin is very old, only its secondary structure may have been conserved, and somatostatin has not been found in *Drosophila*, it was reasoned that SP may be derived from somatostatin, and, hence, the vertebrate somatostatin receptor may correspond to the SP receptor. Indeed, two such *D. melanogaster* genes were identified based on the homology criteria: *AICR2* (*CG13702*) and *star1* (*CG7285*).

To check the hypothesis that one of these genes (or both) is coding for the putative SP receptor, transgenic flies carrying RNAi constructs for these genes (provided by B. Dickson, IMBA, Vienna) were analyzed for the two PMR. The transgenic flies, carrying the RNAi construct for either the *AICR2* or the *star1* gene, were crossed to different Gal4-driver lines: elavGa4, actGal4, tubGal4, and hsGal4, respectively.

Since the SP-binding protein, which is believed to represent the SP receptor, is localized in the CNS, the UAS-RNAi constructs were driven by elavGal4. ElavGal4 is expressed only in the neuronal cells; hence, the putative SP receptor should be switched off. Flies with the following genotypes: “elavGal4; UAS-hairpin for *AICR2*” and “elavGal4; UAS-hairpin for *star1*”, were viable and, therefore, tested for the two PMR. The females, carrying activated RNAi constructs for both genes, demonstrated the rate of receptivity and oviposition comparable to control flies (see Table 2, Fig. 5 - 8). Since one cannot exclude that elavGal4 was not efficient enough to switch off the expression of the *AICR2* or the *star1* genes, other Gal4 drivers were tested.

When the UAS-RNAi was activated by either actGal4 or tubGal4, both leading to constitutive and ubiquitous expression of the RNAi construct, animals from both lines were not viable (Table 2).

The same effect was observed when the UAS-RNAi constructs for both genes were activated by hsGal4 ("hsGal4; UAS-hairpin for *AICR2*" and "hsGal4; UAS-hairpin for *star1*" lines), and the heat shock was given throughout the life span of the animals (from early embryogenesis till adulthood, Table 2). Since the binding protein of SP is expressed in the CNS only in adult females (Ding et al., 2003), the next experiment was carried out in the following manner. Animals "hsGal4; UAS-hairpin for *AICR2*" and "hsGal4; UAS-hairpin for *star1*" were not treated at the embryonic, larval and first part of the pupal stages. The heat shock was applied at the last pupal stage and was carried on in adults. The animals treated this way were viable (Table 2) and tested for the two PMR.

Genotypes	**elavGal4**	**actGal4**	**tubGal4**	**hsGal4[a]**	**hsGal4[b]**
UAS-hairpin for *AICR2*	no phenotype	not viable	not viable	not viable	viable, egg production is strongly reduced
UAS-hairpin for *star1*	no phenotype	not viable	not viable	not viable	viable, egg production is strongly reduced

Table 2 Phenotypes observed for the transgenic flies carrying the RNAi constructs for the *AICR2* and *star1* genes, respectively, driven by the following Gal4-drivers: actGal4, tubGal4, elavGal4 and hsGal4 ([a] heat shock was given throughout the life span, [b] heat shock was given at the last pupal stage and during adulthood).

To test the two PMR in viable "hsGal4; UAS-hairpin for *AICR2*" and "hsGal4; UAS-hairpin for *star1*" flies (the heat shock was given at the last pupal stage and in the adulthood), 5 d old virgins were mated to wt males. On the next day after mating the egg laying rate and the rejection behavior of those females were analyzed. As control flies for the oviposition and receptivity tests, 5 d old virgins with the following genotypes: "*yw*; UAS-hairpin for *AICR2*" and "*yw*; UAS-hairpin for *star1*", were taken up into the analysis. To exclude possible artifacts resulting from the heat shock, the control animals were exposed to the heat shock as well at the last pupal stage and in the adult. Although the expression of RNAi for *AICR2* and *star1* genes at the adult stage did not affect the rejection behavior of the females (Fig. 5, Fig. 7), the egg laying rate was dramatically reduced (see Table 2, Fig. 6 and Fig. 8). Since the SP receptor might affect both PMR (Kubli, 2003), and these results clearly show that none of these genes is involved in the regulation of both PMR, none of them is the SP receptor. According our results, *AICR2* and *star1* genes might play a role in the maintenance of the egg laying machinery. Thus, these genes could be downstream components of SP response cascade which are involved in the control of oviposition, but not in the rejection behavior of the female (Fig. 3). According to our microarray results described below (3.2.1), the transcription of these genes is not affected by the presence of SP (at least 4 h after mating). Thus, *AICR2* and *star1* are very likely involved in the establishment of the egg laying machinery by a mechanism independent of SP response cascade.

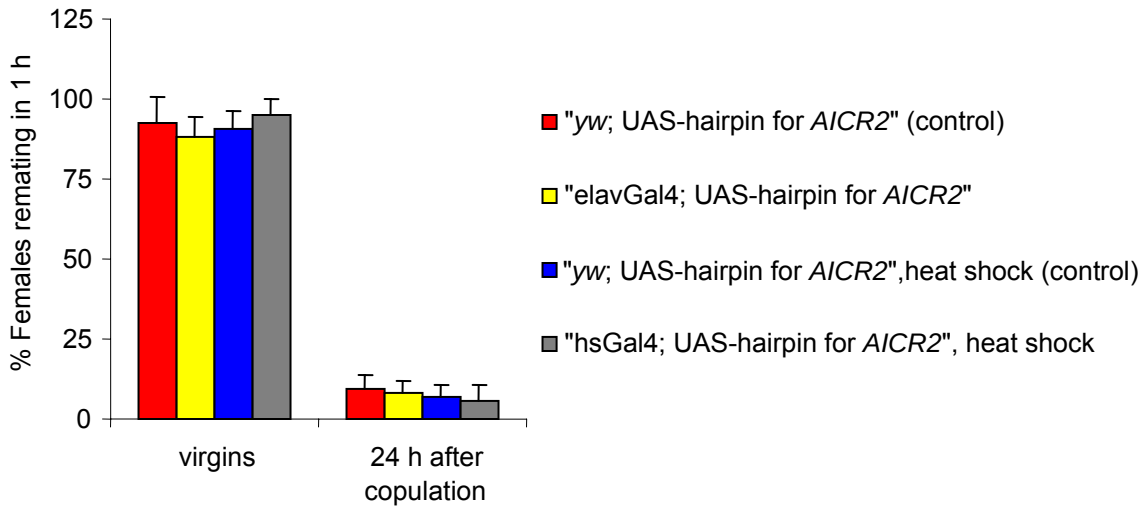

Fig. 5 Receptivity of females of different genotypes mated with wt males. Females used for the test: “elavGal4; UAS-RNAi for *AICR2*”, “*yw*; UAS-RNAi for *AICR2*”, and “hsGal4; UAS-RNAi for *AICR2*”. Flies “*yw*; UAS-RNAi for *AICR2*”, without heat shock application, were used as the control for the test of “elavGal4; UAS-RNAi for *AICR2*” flies. Flies “*yw*; UAS-RNAi for *AICR2*”, with heat shock application, were used as the control for the test of “hsGal4; UAS-RNAi for *AICR2*” flies. The first heat shock was applied at the last pupal stage and was carried on for adults. Standard deviations are indicated.

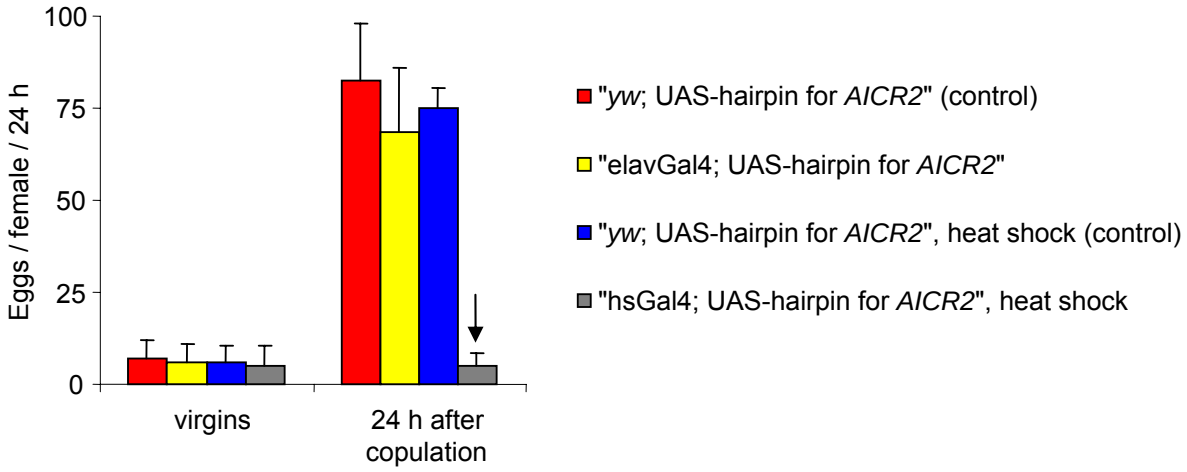

Fig. 6 Oviposition of females of different genotypes mated with wt males. Females used for the test: “elavGal4; UAS-RNAi for *AICR2*”, “*yw*; UAS-RNAi for *AICR2*”, and “hsGal4; UAS-RNAi for *AICR2*”. Flies “*yw*; UAS-RNAi for *AICR2*”, without heat shock application, were used as the control for the test of “elavGal4; UAS-RNAi for *AICR2*” flies. Flies “*yw*; UAS-RNAi for *AICR2*”, with heat shock application, were used as the control for the test of “hsGal4; UAS-RNAi for *AICR2*” flies. The first heat shock was applied at the last pupal stage and was carried on for adults. The expression of RNAi for *AICR2* gene at the adult stage dramatically reduced the egg laying rate (indicated with arrow). Standard deviations are indicated.

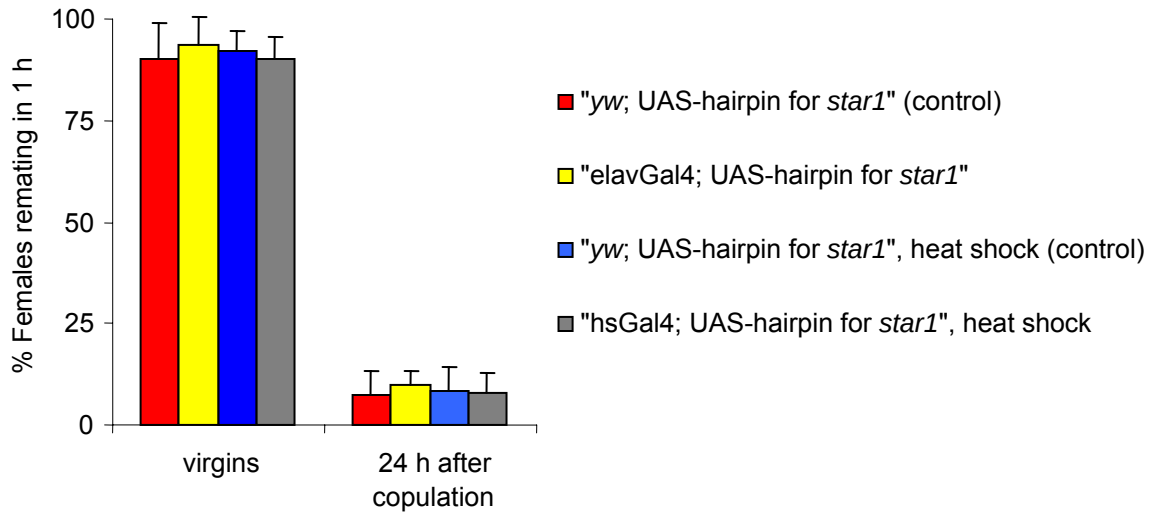

Fig. 7 Receptivity of females of different genotypes mated with wt males. Females used for the test: "elavGal4; UAS-RNAi for *star1*", "*yw*; UAS-RNAi for *star1*", and "hsGal4; UAS-RNAi for *star1*". Flies "*yw*; UAS-RNAi for *star1*", without heat shock application, were used as the control for the test of "elavGal4; UAS-RNAi for *star1*" flies. Flies "*yw*; UAS-RNAi for *star1*", with heat shock application, were used as the control for the test of "hsGal4; UAS-RNAi for *star1*" flies. The first heat shock was applied at the last pupal stage and was carried on for adults. Standard deviations are indicated.

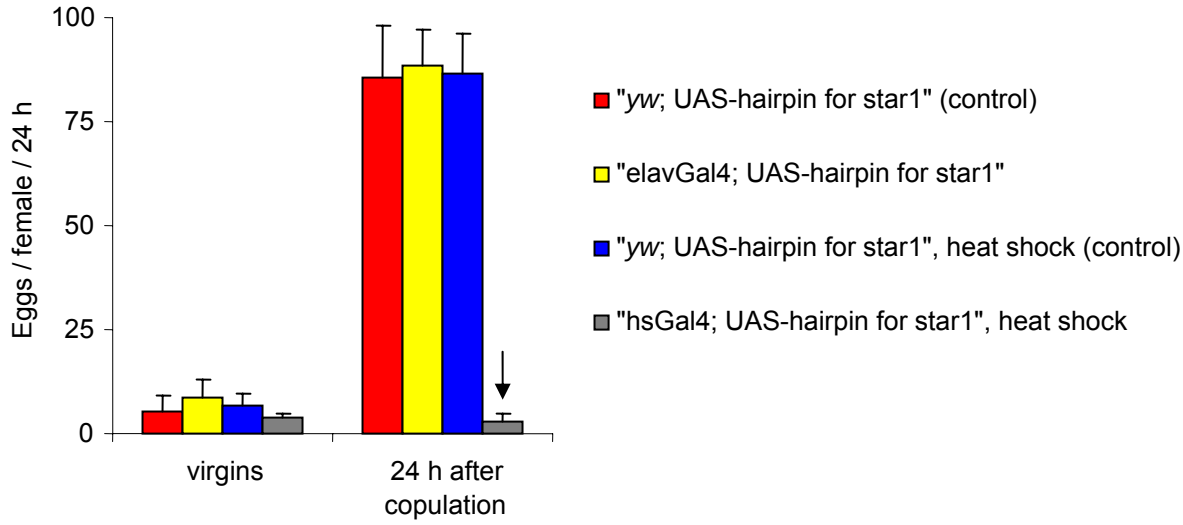

Fig. 8 Oviposition of females of different genotypes mated with wt males. Females used for the test: "elavGal4; UAS-RNAi for *star1*", "*yw*; UAS-RNAi for *star1*", and "hsGal4; UAS-RNAi for *star1*". Flies "*yw*; UAS-RNAi for *star1*", without heat shock application, were used as the control for the test of "elavGal4; UAS-RNAi for *star1*" flies. Flies "*yw*; UAS-RNAi for *star1*", with heat shock application, were used as the control for the test of "hsGal4; UAS-RNAi for *star1*" flies. The first heat shock was applied at the last pupal stage and was carried on for adults. The expression of RNAi for *star1* gene at the adult stage dramatically reduced the egg laying rate (indicated with arrow). Standard deviations are indicated.

3.1.2 Microarray experiment based on the developmental regulation of the SP receptor

This experiment was based on the fact that the expression of a gene coding for the receptor of SP is very likely developmentally regulated in the CNS. In freshly eclosed heads of females there is no binding of AP-SP fusion protein to the receptor of SP, in contrast, in heads of sexually mature females a strong signal was obtained (Ding et al., 2003; Ottiger et al., 2000). The transcription level of the gene encoding the SP receptor was assumed to correlate with the presence of the SP receptor. Hence, to find new candidate genes for the SP receptor in *D. melanogaster*, the microarray technique based on the assumption of a developmental regulation of the SP receptor encoding gene was used. The expression patterns of 13500 genes presented on Affymetrix gene chips were monitored. Age-related changes in transcript levels were measured by comparing RNA extracted from the heads of 3 d old wt females with RNA extracted from the heads of wt females just after eclosion. Three replicates have been done for each time point by using samples of RNA isolated independently. The following criteria were used to define developmentally regulated genes: the t-test, $p = 0.05$; the expression pattern of a gene had to change more than 2-fold; a gene had to be up-regulated in the heads of 3 d old females in comparison to just eclosed females; and the flag restriction (genes that had at least in 2 out of 3 samples “P” (Present) flags for 3 d old flies were accepted; for details see 2.3.4). A set of replicates with RNA isolated from flies immediately after eclosion was not involved in the flag restriction, because the gene coding for the SP receptor is assumed not to be expressed at this developmental stage, thus it would not be surprising if it had an “A” (Absent) flag for this time point (“A” flag means that the signal for perfect match is almost the same as for mismatch).

Finally, 331 genes were chosen for further analysis. Approximately half of these genes encode proteins with unknown functions. Since the cAMP level influences the two post-mating responses, and, thus, the SP receptor might be a GPCR, the obtained list of developmentally regulated genes was checked for the presence of GPCR coding genes. One GPCR coding gene, encoding the Gonadotropin-releasing hormone receptor (AKH receptor in *D. melanogaster*; gene: *GRHR* = *DAKHR* = FBgn0025595), was identified by this microarray experiment. *GRHR* demonstrated a 5.5-fold up-regulation in the heads of 3 d old females compared to freshly eclosed females. Thus, GRHR was further analyzed as a putative receptor for SP (see 3.1.4). However, I could not fully exclude other developmentally regulated genes as SP receptors. Thus, to narrow down this list of genes, an LC-MS/MS based approach was performed (see 3.1.3).

3.1.3 Proteomics approach

Strategy of the experimental procedure

The next step in the SP receptor isolation was a proteomics approach. Under non-denaturing conditions SP was shown to bind to the "SP receptor" (Ding et al., 2003). This previous work was expanded by using the LC-MS/MS technique allowing to sequence 20 to 50 proteins from one sample at the same time. The idea behind this experiment was to identify a list of proteins, and compare it with the previously obtained microarray data (see 3.1.2), hence, to narrow down the list of possible candidates for the role of SP receptor.

A schematic representation of the experimental procedure is presented in Fig. 9. Briefly, membrane and cytosolic extracts were prepared from heads and abdomen, respectively, from 3-day old (sexually mature) virgin females. The extracts were separated on Native PAGE (each sample was loaded into two neighboring wells), and afterwards the gel was cut into two pieces to carry out further analysis. One part of the gel was used to identify the localization of the signal representing SP binding to the "SP receptor" after Native PAGE separation of the membrane proteins. For this purpose, the gel was transferred to a nitrocellulose membrane and incubated with an AP-SP_{8-36} probe. It has been shown that AP-SP_{8-36} (truncated SP) has the same biological activity and binding pattern in the nervous system and the genital tract as the full-length protein, but produces a lower background (Ding et al., 2003). On the affinity blots, two different bands showed up for the membrane extracts from heads and abdomen, respectively (Fig. 10). Since it was not possible to reproduce this experiment under denaturing conditions, one could not conclude whether the different bands are based on different protein sequences or on protein modification (Ding et al., 2003).

The second part of the same gel was stained with Coomassie Brilliant Blue (Fig. 10) and the obtained band pattern was compared with the identified signals on the nitrocellulose membranes. In both cases most of the membrane proteins were concentrated in one band (extracts from heads and from abdomen). Then, appropriate bands were cut off from the gel and polypeptides extracted from these bands were analyzed using LC–MS/MS. The database search of the sequenced pieces resulted in identification of the corresponding *Drosophila* proteins with a certain likelihood. To corroborate our data, this experiment has been independently carried out twice for the head membrane protein fraction and for the abdomen fraction. The data are believed to be reliable when several short aa sequences are identified inside of a full-length protein sequence.

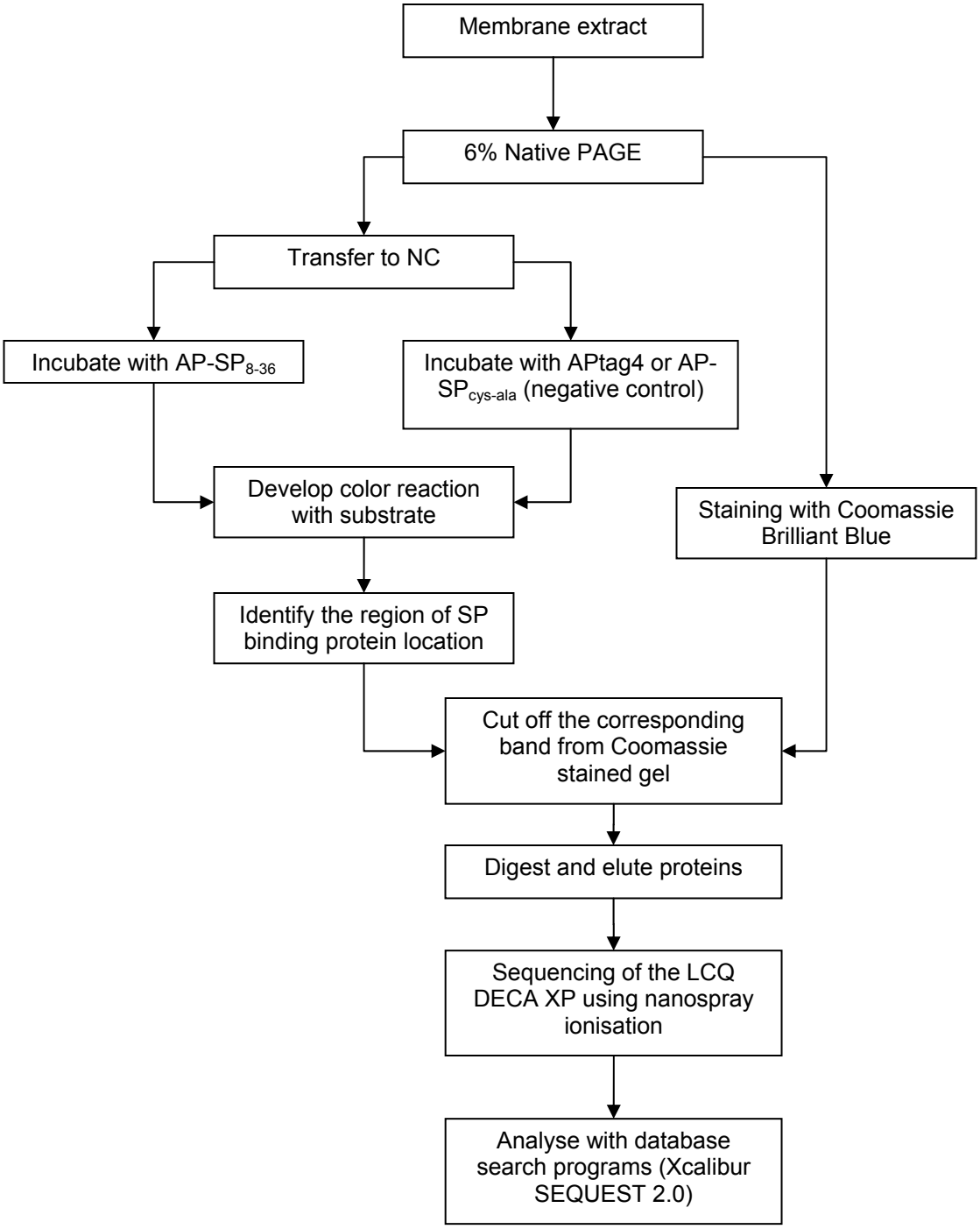

Fig. 9 Schematic representation of the experimental procedure for the proteomics approach. See the text for details.

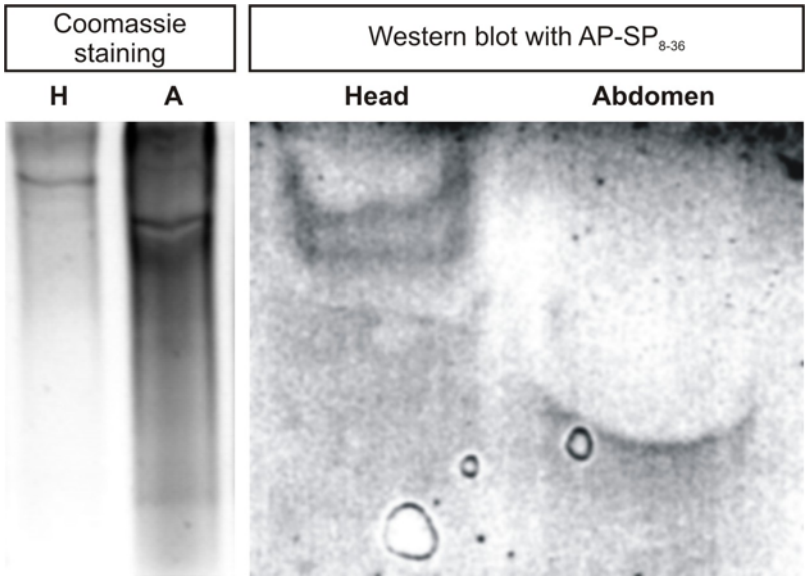

Fig. 10 Coomassie stained 6% Native PAGE and affinity blot of Sex-Peptide binding proteins. Membrane extracts were separated on a nondenaturating PAGE, and either stained with Coomassie Brilliant Blue, or transferred to a nitrocellulose membrane and probed with AP-SP_{8-36} fusion protein. The AP-SP_{8-36} fusion protein binds to two molecularly different proteins in the membrane extracts prepared from heads and abdomen, respectively. Membrane extract from the head of virgins (**H**), membrane extract from the abdomen of virgins (**A**).

Are the affinity blot results artifacts?

To be sure that the obtained binding is indeed SP specific, relevant negative controls had to be made based on the binding specificity of SP to its receptor and also on the fact of the stage-dependent manner of the SP receptor expression. First, to exclude the possibility that observed signals represent the background binding due to the presence of alkaline phosphatase, APtag-4 was used as one of the negative controls (Flanagan and Cheng, 2000). Other controls used in this experiment were chosen based on the following facts. Different fragments of SP bind differentially to the target sites (Ding et al., 2003), e. g. neuronal binding is dependent on the intact C-terminal part of the peptide and the disulfide bridge. Truncated SP with the last cys substituted by ala (SP_{8-36A}) does not form the disulfide bridge and is not able to bind to its target in the CNS (Ding et al., 2003). Taking an extract of membrane proteins from freshly eclosed females as a negative control was based on the fact of developmental regulation of the SP neuronal binding partner. The neuronal target sites cannot be detected until immediately after eclosion, while 36 h after eclosion labeling is at the same level of intensity as in 5 d old sexually mature females (Ding et al., 2003). Thus, to avoid artifactual binding, the following negative controls were used in this experiment: APtag-4 and AP-SP_{8-36A} probes, and also an extract of membrane proteins from freshly eclosed females. Binding signals were obtained for all these negative controls at the same level as for SP (Fig. 11), and they corresponded to the single band

which was observed on the Coomassie Brilliant Blue stained gel (Fig. 10). These finding clearly indicated that the previously observed binding of SP to its receptor might be an artifact. Thus, this approach was not suitable for the purpose of fishing the SP receptor, and it was stopped at this point. As a consequence, it was not possible to narrow down the microarray data.

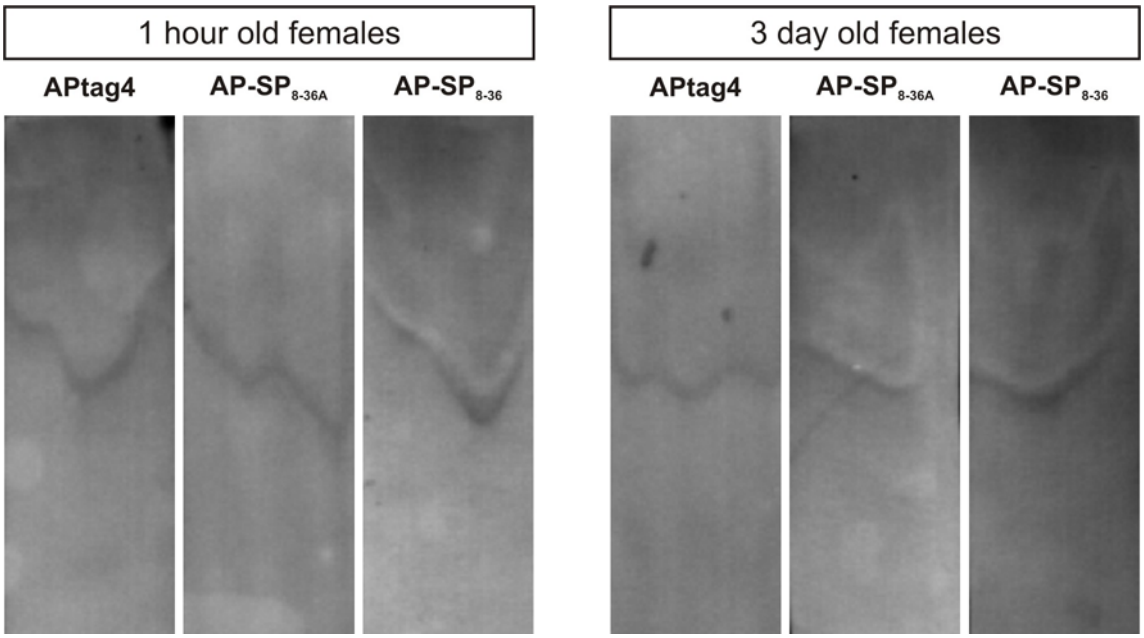

Fig. 11 Affinity blot of Sex-peptide binding proteins of membrane extracts from the heads of 1 h and 3 d old virgins, respectively. The NC membrane was incubated with 20 nM APtag4, 20 nM AP-SP_{8-36A} or 20 nM AP-SP_{8-36}, respectively.

3.1.4 Analysis of the GRHR transposon insertions

GRHR is a putative candidate SP receptor based on the results of the previously described microarray experiment (see 3.1.2). The gene *GRHR* has been described as a gene coding for a receptor that is structurally related to the Gonadotropin-Releasing Hormone (GnRH) receptors from vertebrates (Hauser et al., 1998). The GRHR in *Drosophila* is activated by the AKH hormone that takes part in the mobilization of sugars and lipids from the insect fat body during energy-requiring activities (Staubli et al., 2002). Based on the fact that certain GPCRs exhibit multiple ligand sensitivity (Park et al., 2002), I hypothesized that the GRHR may have at least two different ligands: AKH and SP.

To check whether the disruption of *GRHR* abolishes the PMR, an analysis of available transposon insertions was performed. Two lines carrying transposon insertions in *GRHR* are available from the Bloomington centre. One of the insertions, PBac, $GRHR^{c03956}$, also named

11320 (Thibault et al., 2004), is located in the second intron of *GRHR*. The second insertion, Pge, $GRHR^{EY11371}$, also named 20304 (Bellen et al., 2004), is located in the first intron of *GRHR* (Fig. 12). To check whether these insertions disrupt the gene, RT-PCR was performed for cDNA synthesized from the RNA extracted from 11320, 20304 and *yw* (as positive control) flies (Fig. 13). The combination of the following primers was used for RT-PCR: the primer designed from the first exon (pr-GRHR-1ex) or the primer from the second exon (pr-GRHR-2ex) was combined with the primer designed from the third exon of *GRHR* (pr-GRHR-3ex). In the case of *yw* flies, a 580 bp PCR product was synthesized from pr-GRHR-1ex and pr-GRHR-3ex, and a 360 bp PCR product was synthesized from the pr-GRHR-2ex and pr-GRHR-3ex (Fig. 13). While the same PCR fragments were obtained for the 20304 flies, there were no detected PCR products for the 11320 flies (Fig. 13). Based on these data, I concluded that the 11320 line was a mutant for *GRHR*, while the 20304 insertion does not disrupt the gene. Thus, the line 20304 was used as a control for the further analysis.

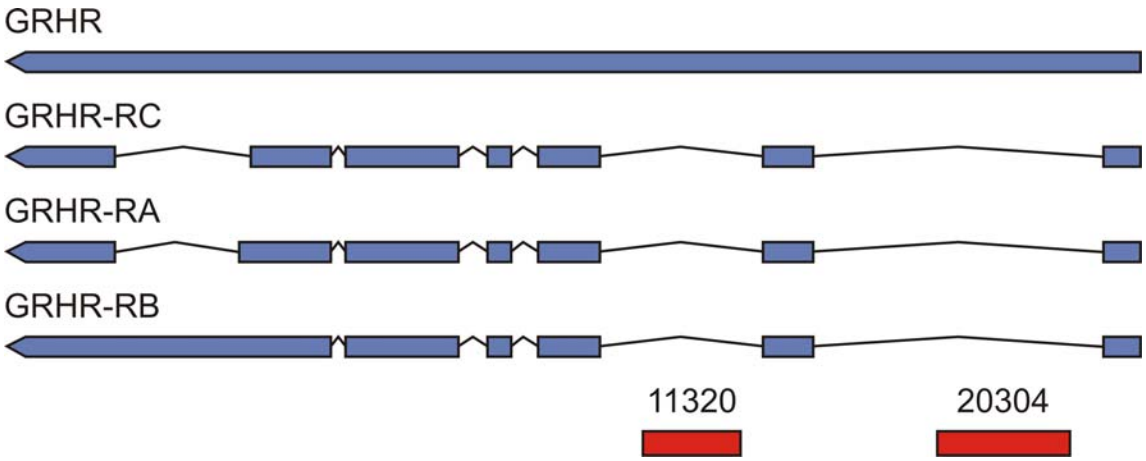

Fig. 12 GRHR-RA, GRHR-RC and GRHR-RB are splice variants of the *GRHR* gene. 20304 and 11320 are alignments of the 20304 and 11320 P element insertion flanking sequences into *GRHR* genomic DNA. 20304 is a P element insertion in the first intron, and 11320 is an insertion in the second intron of *GRHR* (FlyBase Genome Browser: *D. melanogaster*, release 4.1; Feb 2005).

To check the hypothesis that GRHR is the receptor for SP, flies carrying the 11320 transposon insertion were analyzed for the two post-mating responses. 11320 females were mated with wt males, and 24 h after copulation the egg laying rate and receptivity were analyzed. The oviposition test was additionally done 48 h after mating. *yw* and 20304 females mated with wt males were used as a positive control. The results of the oviposition test are presented in Fig. 14. Flies with a disrupted *GRHR* gene (11320) lay fewer eggs than control flies, but still significantly more eggs than virgins. Therefore, 11320 flies did not show the phenotype expected for the SP receptor. Moreover, the rejection behavior for 11320 flies is the same as for *yw* (Fig. 15), which indicates, that *GRHR* does not have a role in the regulation of both post-mating responses. Such

a decreased number of laid eggs could be for example explained by the fact that *GRHR*, as a receptor for adipokinetic hormone, is involved in the energy metabolism. The disruption of the gene could lead to general metabolic problems in females and to a lack of resources needed for the egg production.

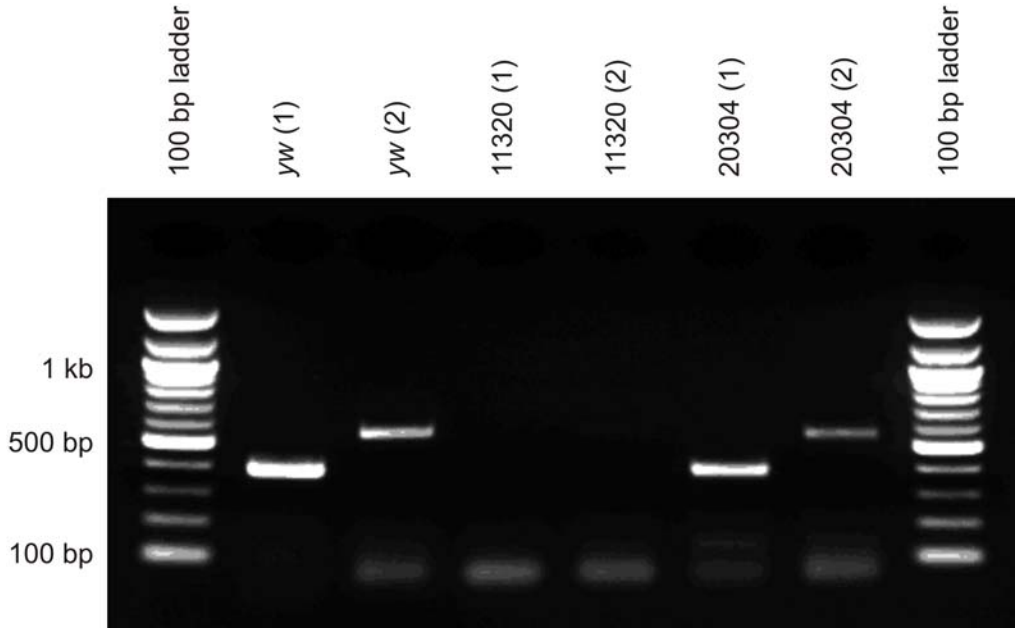

Fig. 13 RT-PCR from cDNA prepared from *yw*, 11320 and 20304 flies. Total RNA was prepared from flies of different genotypes, and was used as a template for cDNA synthesis followed by PCR analysis. ***yw* (1)** 360 bp length PCR fragment synthesized from GRHR-2ex and pr-GRHR-3ex from cDNA of *yw* flies. ***yw* (2)** 580 bp length PCR fragment synthesized from pr-GRHR-1ex and pr-GRHR-3ex from cDNA of *yw* flies. **11320 (1)** PCR fragment synthesized from pr-GRHR-2ex and pr-GRHR-3ex from cDNA of 11320 flies. **11320 (2)** PCR fragment synthesized from pr-GRHR-1ex and pr-GRHR-3ex from cDNA of 11320 flies. **20304 (1)** 360 bp length PCR fragment synthesized from pr-GRHR-2ex and pr-GRHR-3ex from cDNA of 20304 flies. **20304 (2)** 580 bp length PCR fragment synthesized from pr-GRHR-1ex and pr-GRHR-3ex from cDNA of 20304 flies.

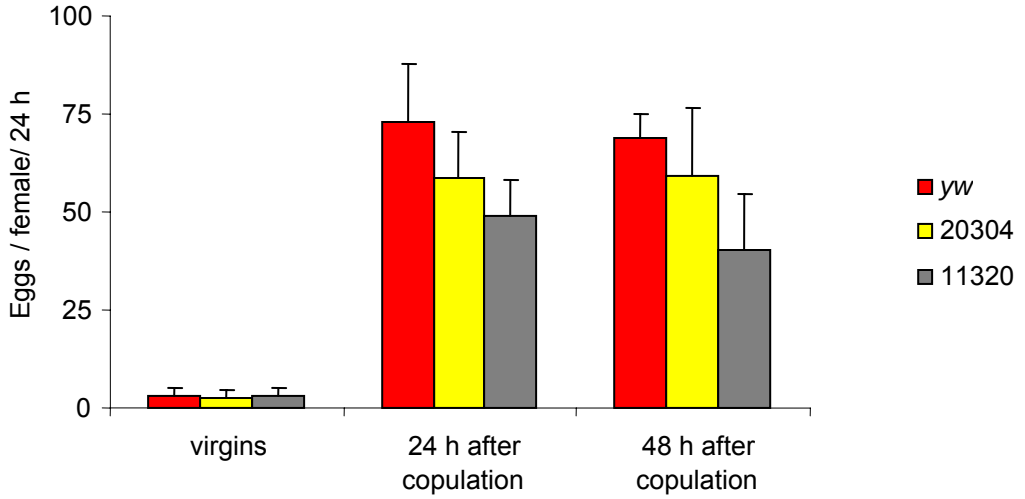

Fig. 14 Oviposition of females of different genotypes: *yw*, 20304 and 11320, mated with wt males. Number of eggs laid per female in 24 h was counted for virgin flies and for females 24 and 48 h after mating. Standard deviations are indicated.

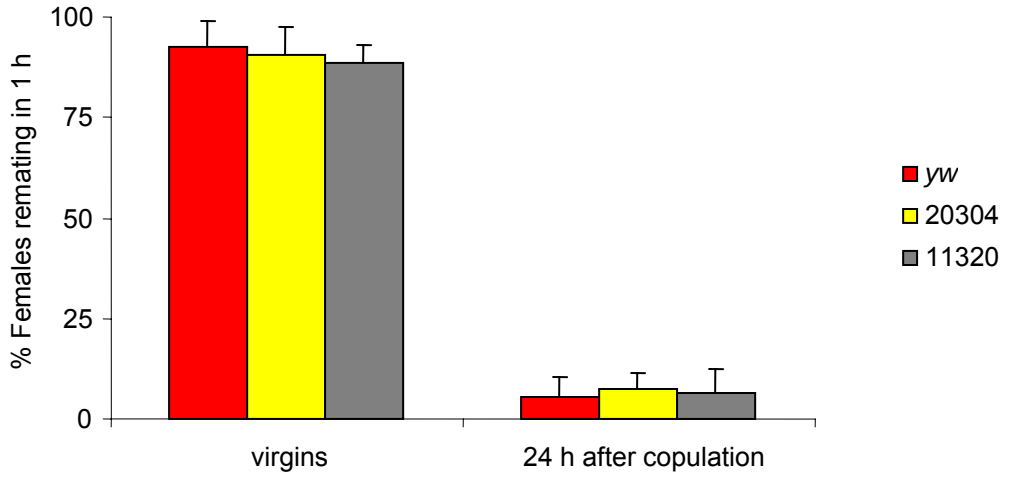

Fig. 15 Receptivity of females of different genotypes: *yw*, 20304 and 11320, mated with wt males. Standard deviations are indicated.

3.1.5 OTK is a new candidate SP receptor based on the “DUALmembrane system” approach

To find new candidate genes for the role of the SP receptor the following approach was initiated. Since most of the techniques available so far (classical two-hybrid screen, cDNA library screens) assay the interaction with a transmembrane protein under unnatural conditions (SP with a putative transmembrane receptor), i.e. outside the membrane, they are not optimal for the SP receptor screening. Therefore, the “DUALmembrane system” (or MbYTH) of DualSystems Biotech was used to find SP-binding proteins by screening two cDNA libraries prepared from the heads of 3 d old females. The “DUALmembrane system” uses the split-ubiquitin system as its basis to detect interactions between integral membrane proteins or cytosolic proteins. Unlike the “Yeast Two-Hybrid system” the MbYTH system is not dependent on the localization of bait and prey in the nucleus. In this advanced system ligand-receptor interactions can be studied in the membrane, i.e. under more realistic conditions than usual (for details see 5. Appendix). Furthermore, with the MbYTH system it is possible to screen full-length, integral membrane proteins, as well as membrane-associated proteins.

Bait cloning: SP is fused to a transmembrane receptor and exposed outside a cell

Since SP is an extracellular peptide pheromone, it has to be anchored to a known transmembrane protein to apply the MbYTH system. Moreover, SP should be anchored in such a way that it’s C-terminus is exposed to the lumen of the cell and can easily interact with its binding partners. To generate the bait, full length SP peptide was N-terminally fused to the full length yeast membrane glycoprotein Sec12. This fusion of cDNAs was cloned into the pNDB1 vector (see 2.7.1) containing the weak Cytochrome-C oxidase (*CYC1*) promoter, which provides low-level expression of the bait fusion protein, a multicloning site (MCS), the C-terminal (Cub) domain of yeast ubiquitin fused in frame to an artificial transcription factor consisting of *Escherichia coli* LexA (DNA-binding protein), and the *Herpes simplex* virus transcriptional activator VP16 (all together called TF), as well as the *LEU2* gene for selection in yeast. Sec12 was N-terminally fused to TF-Cub, thus generating TF-Cub-Sec12-SP. The expression of the vector resulted in the formation of the transmembrane fusion protein where SP with its free C-terminus was exposed outside the cell, and the N-terminus of SEC12 was fused to the Cub and exposed intracellularly (this step was done by DualSystems Biotech).

Prey cloning and screening of two cDNA libraries

Transmembrane proteins are classified in different ways. One classification is based on the orientation of their N- and C-terminal parts. A type I integral membrane protein presents its N-terminus on the extracellular side, and it's C-terminus to the cytosol; a type II class protein is integrated in the membrane in the reverse way: the C-terminus is outside of the cell and the N-terminus is inside. All GPCRs belong to the type I transmembrane receptors. Since the SP receptor may be a GPCR, but the possibility that it could be a different type of the membrane protein cannot be excluded, the MbYTH system was geared to screen both types of integral membrane proteins. Therefore, two different cDNA libraries were prepared from the RNA extracted form 3 d old wt females and screened for binding with SP (done by DualSystems Biotech): a NubG-x (carboxy terminus of protein is outside the cell, type II membrane proteins) and a x-NubG (amino terminus of the protein outside the cell, type I membrane proteins) cDNA libraries. NubG is the N-terminal fragment of ubiquitin where isoleucine was substituted with glutamine. A cDNA library of $3.0x10^7$ complexity was obtained for the NubG-x collection, and a cDNA library of $2.6x10^6$ complexity was obtained for the x-NubG collection. These fly head cDNA libraries were transformed into the bait-bearing strain and interactors were selected by the presence of β-galactosidase activity. Whereas no clones were selected from the $1.8x10^6$ transformants of the NubG-x library, one β-galactosidase positive clone was detected from $2.6x10^6$ transformants of the x-NubG library. Hence, as a result of the screening, one interaction was observed for SP: the off-track receptor tyrosine kinase (OTK). To confirm that this interaction is not a false positive, further verification by pairwise interaction analysis is needed (*otk* cDNA with *SP* cDNA).

***otk* loss-of-function mutation and its interaction with SP**

OTK was identified as an axon guiding protein in the embryo interacting with plexin and semaphorins. Since it is expressed in the nervous system, it is a valid candidate for a SP receptor. Since, the *otk* loss-of-function mutants are lethal; a "transheterozygous" genetic test was applied to check if OTK is involved in the PMR of the female and if it is a valid SP receptor candidate. For most proteins, reducing the gene dose to a single copy leads to a mild defect in a phenotype. However, reducing the gene dose of two different proteins may generate a mutant phenotype if these proteins function together. Thus, embryos lacking one copy of each of both *otk* and *PlexA* exibit the same mutant phenotype as seen in the single homozygous mutants (Winberg et al., 2001).

Since the injection experiments with synthetic SP revealed that the SP receptor is sensitive to SP concentration (Schmidt et al., 1993a), and the minimal amount of SP able to induce the PMR is 0.6 pmol, the following experiment was performed. Females heterozygous for the otk^3 allele (otk^3 -/+; Winberg et al., 2001), which is *otk* loss-of function, were injected with different concentration of SP (0.5 pmol, 0.6 pmol, 0.7 pmol and 1 pmol). Four to six hours later, the ovulation test was done. In the case of OTK being the SP receptor, I expected to observe the loss of function phenotype of the SP receptor. In other words, the females heterozygous for the otk^3 allele should not react to the critical SP concentration, 0.6 and 0.7pmol. In fact, the ovulation test showed that otk^3 -/+ females have the same sensitivity to SP as wt females. Furthermore, both of these strains did not react to 0.5 pmol SP and showed a normal ovulation rate for higher amounts of injected SP (data not shown). Although the "transheterozygous" genetic test showed that there is no interaction between SP and OTK, I can not conclude that OTK is not a receptor for SP. The reason is that in the case of SP/receptor interaction the manner of binding and activation of the downstream components is not clear. The experiment performed above was based on the assumption that the SP/OTK interaction would work in the same mode as the Plexin/OTK complex. To demonstrate that OTK is indeed a receptor for SP, more experiments are needed.

3.2 Downstream components of the SP response cascade

3.2.1 Genome-wide analysis of SP responses

To determine which genes are regulated by SP in the head and in the abdomen to serve the two PMR, I performed a genome-wide expression analysis aimed at identifying all transcripts from *D. melanogaster* that exhibit SP-dependent expression patterns. This experiment can be performed due to the accessibility of the SP^0 strain as well as some other transgenic lines based on the SP^0 background (Liu and Kubli, 2003; Peng et al., 2005a).The microarray analysis was performed independently for heads and abdomen of females, since there are two binding sites for the SP in the female body: the CNS and the genital tract (Ding et al., 2003; Ottiger et al., 2000), which likely correspond to two different SP binding proteins (Ding et al., 2003). To get the earliest genes that are controlled by SP, 4 h after copulation was taken as a time point for the analysis. Four hours is a time point at which early genes that are controlled by SP should show a differential expression pattern. These genes may give a hint about the nature of the SP-signaling cascade and the receptor of SP. At this time point females already demonstrate their rejection behavior and increased ovulation, but it is still too early to observe an increased egg laying rate. Knowledge of the genes controlled by SP in the heads and in the abdomen of mated females, respectively, and their comparison might help us to understand the SP response cascade and the role of these two SP binding proteins and their functions.

To obtain flies for the microarray experiment, 5 d old wt virgin females were mated with 5 d old males of the following genotypes: SP^0 (0325/Δ130) and $Control^{SP}$ (0416/Δ130), and $SP^{\Delta 2-7}$ (Cd; 0325/Δ130) and $Control^{\Delta 2-7}$ (C0; 0325/Δ130). For this purpose, 1 female and 2-3 males were put in one single tube. Females mated at about the same time (+/- 10 min) were collected in a single tube. Four hours after copulation, heads and abdomen were separately cut off from the females and collected.

3.2.1.1 Genes regulated by SP in the head

Genes that are controlled by SP in the head were identified by comparing the pattern of mRNA expression in the heads of females mated with SP^0 and $Control^{SP}$ males, 4 h after mating. Thirty-four genes were shown to be significantly regulated by SP: 11 of them are up-regulated and 23 are down-regulated. Among these genes, 8 are involved in metabolism (Table 3), 4 in proteolysis

(Table 4), 7 in signal transduction, regulation of transcription, and transport (Table 5). Finally, 15 genes are either of unknown function or with a function that does not correlate with any of the listed groups (Table 6).

SP is involved in "metabolism control"

After copulation the egg production level is drastically increased, and, thus, metabolism might undergo significant changes. Whereas virgin females lay around 2 eggs per day, females after copulation increase the amount of laid eggs up to 80 eggs per day (Chen et al., 1988; Soller et al., 1997; Soller et al., 1999). This means that a female has to mobilize her nutritional and energy sources after mating to produce as many eggs as possible. Genes involved at various steps of several metabolic pathways demonstrated SP-related changes at their transcript levels (Table 3). These include genes involved in: carbohydrate (*CG8845*, *CG9357*, *CG10531* and *CG6004*), lipid (*CG4825*, *CG4500* and *CG5966*), and amino acid metabolism (*CG5840*). Interestingly, all genes involved in carbohydrate metabolism are down-regulated by SP at this time point. Some genes involved in lipid metabolism are up- as well as down-regulated. One gene involved in amino acid metabolism is up-regulated by SP. Based on the obtained results; I can conclude that 4 h after mating metabolism in the head is rather suppressed by SP than activated.

SP-regulated genes involved in proteolysis and peptidolysis in the head

Among the genes displaying changes in mRNA expression dependent on SP in the head, 3 genes coding for proteases (*λTry*, *γTry* and *CG10472*), and 1 gene coding for a serine-protease inhibitor (*m1*) were detected (Table 4). All these genes are down-regulated by SP. Their function in reproductive and in other processes is unknown.

Metabolism	Gene name	Fold change	Function	Additional information
Carbohydrate metabolism	*CG8845*, *CG31956, pgant4*	↓ 0.4	Polypeptide N-acetylgalactosaminyltransferase activity	Glycoprotein biosynthesis (www.flybase.org)
	CG9357	↓ 0.4	Polysaccharide metabolism	Chitin binding domain, tachycitin (www.flybase.org)
	CG10531	↓ 0.3	Hydrolase, chitinase, polysaccharide metabolism, signal transduction	-
	CG6004	↓ 0.27	Peritrophin-like (structural protein)	Chitin binding domain, tachycitin (www.flybase.org)
Lipid metabolism	*CG4825*	↑ 2.2	CDP-diacylglycerol-serine O-phosphatidyltransferase activity	-
	CG4500	↓ 0.46	Long-chain-fatty-acid-CoA ligase activity	Mesoderm development (Furlong et al., 2001)
	CG5966	↓ 0.31	Triacylglycerol lipase activity	-
Amino acid metabolism	*CG5840*	↑ 2	Pyrroline 5-carboxylate reductase; aminoacid biosynthesis	-

Table 3 "Metabolism-related" genes whose transcript levels in the head are changed by the presence of SP (4 h after copulation). Gene expression in females mated with SP^0 males was assumed as a background control value. "Fold change" was calculated for the expression level of the same gene in females mated with $Control^{SP}$ males related to the control value of the gene. In the column "Fold change", the level of a gene expression in the females mated with $Control^{SP}$ males is compared with the level in females mated with SP^0 males. The arrow shows either an up-regulation (↑) or a down-regulation (↓) of the gene by SP.

Gene name	Fold change	Function	Additional information
CG12350 (*λTry*)	↓ 0.5	λTrypsin, extracellular	-
CG30031 (*γTry*)	↓ 0.3	γTrypsin , extracellular	-
m1 (E(spl) region transcript m1)	↓ 0.33	Serine-type endopeptidase inhibitor activity	-
CG10472	↓ 0.23	Serine-type endopeptidase activity, chymotrypsin activity	-

Table 4 Genes coding for proteases whose transcript levels in the head are changed by the presence of SP (4 h after copulation). Gene expression in females mated with SP^0 males was assumed as a background control value. "Fold change" was calculated for the expression level of the same gene in females mated with $Control^{SP}$ males related to the control value of the gene. In the column "Fold change", the level of a gene expression in the females mated with $Control^{SP}$ males is compared with the level in females mated with SP^0 males. The arrow shows either an up-regulation (↑) or a down-regulation (↓) of the gene by SP.

SP-regulated genes involved in signal transduction, regulation of transcription and transport in the head

One of the most interesting and conspicuous group of SP-dependent genes includes 7 genes coding for proteins involved in signal transduction, regulation of transcription or transport (Table 5). Further analyses of mutants of these genes and their role in establishment of the PMR might give a hint about the nature of the SP receptor and the SP-signaling cascade.

Other genes regulated by SP in the head

Thirteen genes with unknown functions showed a SP-dependent expression pattern. Among them only 3 genes are activated by SP, the rest is suppressed after mating (Table 6). An interesting fact was that there are 4 SP-dependent genes expressed in the head which encode unknown products but each containing a tachycitin domain: *CG3348*, *CG14645*, *CG14125*, and *CG12726*. As tachycitin is an antimicrobial protein with chitin-binding activity in horseshoe crab hemocytes, these genes could be also involved in the microbial defense in *Drosophila*.

Gene name	Fold change	Function	Additional information
Edl (*mae*, *CG15085*)	↑ 2.7	*mae* encodes a product that directly links the MAPK signaling pathway to its downstream transcription factor targets; regulation of RAS protein signal transduction; regulation of phosphorylation; regulation of transcription, DNA-dependent	MAE, a dual regulator of the EGFR signaling pathway, is a target of the Ets transcription factors PNT and YAN (Vivekanand et al., 2004)
Rala (*CG2849*)	↑ 2.7	*Rala* has RAS small monomeric GTPase activity. It is involved in negative regulation of JNK cascade. *Rala* regulates developmental cell shape changes through the JNK	-
CrebA (*CG7450*)	↑ 2.5	Cyclic-AMP response element binding protein A, RNA polymerase II transcription factor	-
CG17100 (*sticky ch1*)	↑ 2.3	RNA polymerase II transcription factor activity, regulation of transcription	HER subfamily (www.flybase.org)
DrdgBβ (*CG17818*)	↑ 2.1	Phosphatidylinositol transporter activity	Lipid metabolism, vesicle-mediated transport (www.flybase.org)
CG6565	↑ 2	Phosphatidylcholine transporter activity	-
CG33173	↓ 0.39	ATP-binding cassette (ABC) transporter activity	-

Table 5 Genes coding for proteins involved in signal transduction, regulation of transcription and transport whose transcript levels in the head are changed by the presence of SP (4 h after copulation). Gene expression in females mated with SP^0 males was assumed as a background control value. "Fold change" was calculated for the expression level of the same gene in females mated with $Control^{SP}$ males related to the control value of the gene. In the column "Fold change", the level of a gene expression in the females mated with $Control^{SP}$ males is compared with the level in females mated with SP^0 males. The arrow shows either an up-regulation (↑) or a down-regulation (↓) of the gene by SP.

Affymetrix ID	FlyBase ID	Fold change	Additional information
1. Unknown genes			
151094_at	*CG3348* (FBgn0040609)	↑ 5.4	Chitin binding domain, tachycitin (www.flybase.org)
141221_at	*CG8253* (FBgn0034046)	↑ 2.8	-
150846_at	*CG31037* (FBgn0039700)	↑ 2.3	TolB, C-terminal domain, regulator of chromosome condensation RCC1 (www.flybase.org)
153582_at	*CG13101* (FBgn0032084)	↓ 0.5	Putatively involved in development (www.flybase.org)
147525_at	*CG16898* (FBgn0034480	↓ 0.5	-
151164_at	*CG14645* (FBgn0040687)	↓ 0.44	Tachycitin (www.flybase.org)
149478_at	*CG11672* (FBgn0037563)	↓ 0.37	-
148624_at	*CG14125* (FBgn0036232)	↓ 0.33	Chitin binding domain, tachycitin (www.flybase.org)
145009_at	*CG12726* (FBgn0030487)	↓ 0.33	Chitin binding domain, tachycitin (www.flybase.org)
152760_at	*CG1979* (FBgn0026563)	↓ 0.32	-
152167_at	*CG3906* (FBgn0034871)	↓ 0.3	-
150233_at	*CG4783* (FBgn0038756)	↓ 0.27	-
142951_at	*CG7916* (FBgn0028534)	↓ 0.22	-
144310_at	*CG8997* (FBgn0028920)	↓ 0.16	-
2. Others			
147206_at	*Lobe* (*L*, FBgn0033940)	↑ 2	Regulation of imaginal disc growth

Table 6 Unknown genes and others that are regulated by SP in the head (4 h after copulation). Gene expression in females mated with SP^0 males was assumed as a background control value. "Fold change" was calculated for the expression level of the same gene in females mated with $Control^{SP}$ males related to the control value of the gene. In the column "Fold change", the level of a gene expression in the females mated with $Control^{SP}$ males is compared with the level in females mated with SP^0 males. The arrow shows either an up-regulation (↑) or a down-regulation (↓) of the gene by SP.

3.2.1.2 Genes regulated by SP in the abdomen

By comparing the pattern of mRNA expression in the abdomen of females mated with SP^0 males and females mated with $Control^{SP}$ males, 18 genes that exhibited changes in their transcript levels were identified and classified as: immune response genes (8 genes, Table 7), unknown genes (8 genes) and 2 others (Table 8). Most of these genes are up-regulated by SP, except 2 genes with unknown function that are down-regulated.

SP and induction of the immune response in the abdomen

Drosophila is devoid of an adaptive immune system and relies only on innate immune reactions for its defense against pathogens. Two different pathways activate the transcription of antimicrobial peptides: the Toll and the Imd pathway. The Toll pathway controls the resistance to fungal and gram-positive bacterial infections. The Imd pathway is responsible for the defense against Gram-negative bacteria. Different antimicrobial peptides are induced by distinct and overlapping pathways (Hoffmann and Reichhart, 2002). According to our microarray data the immune response is dramatically activated in the abdomen by SP (Table 7). Seven antimicrobial peptides and one peptidoglycan-recognition protein induced by either the Imd or the Toll pathway, are up-regulated by SP: *DptB*, *Dpt*, *Dro*, *AttC*, *AttA*, *Mtk*, *IM1* and *PGRP SB1*. Further, more detailed experiments concerning the SP-dependent activation of the immune response are described in 3.2.2.

Other genes regulated by SP in the abdomen

Several other SP-dependent genes encoding proteins with unknown functions as well as the genes *timeless* (*tim*) and *Rab1* were identified by microarray analysis (Table 8). Since *timeless* was recently shown to be expressed in the peripheral tissue in a non-circadian manner and since it seems to be involved in egg production and mating behavior (Beaver et al., 2002; Beaver et al., 2003), a more detailed analysis was performed for this gene. Null mutations in the clock genes, per^{01} and tim^{01} result in a lower number of eggs laid per couple, and a higher than normal rate of the laying of unfertilized eggs. Moreover, the clock mutants might compensate for their lower reproductive fitness by re-mating more often (Beaver et al., 2002). Thus, based on the described role of *tim* in the reproductive fitness and the proposed higher rate of re-mating for tim^{01} females, I wanted to check the two PMR in tim^{01} females more precisely. The tim^{01} females were mated with wt males and 24 h after mating the egg laying rate and rejection behavior of mated females were monitored. Although, the oviposition of mated tim^{01} females was indeed decreased (Fig. 16), the rejection behavior was the same as for wt mated females (data not shown). Thus, *tim* indeed has

an input in the control of the such physiological changes as increased egg laying rate in the females after mating, but it is not involved in the establishment of the after mating behavioral mode.

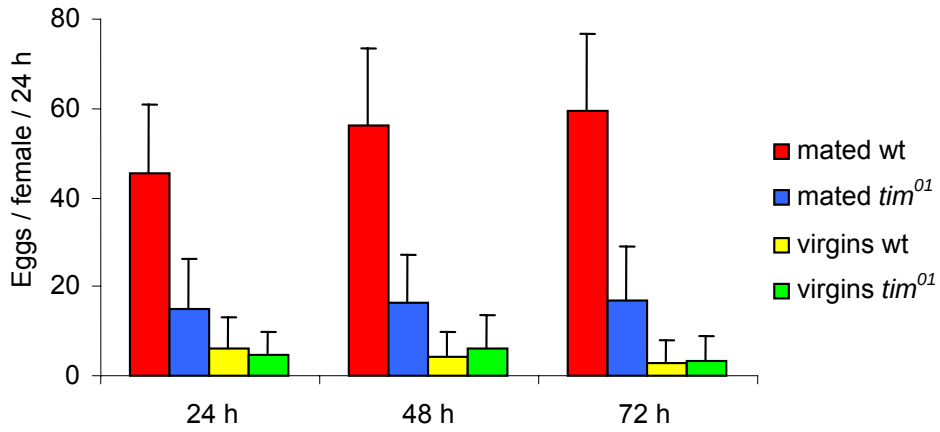

Fig. 16 Oviposition of females of different genotypes: wt and *tim*^{01}, mated with wt males or virgins. Number of egg laid per female in 24 h was counted for virgin flies and for females 24 and 48 h after mating. Standard deviations are indicated.

Gene name	Fold change	Function	Additional information
DptB (*Diptericin B*, *CG10794*)	↑ 3.7	Antibacterial humoral response	Toll and Imd pathway (De Gregorio et al., 2002)
Dpt (*Diptericin*, *CG12763*)	↑ 3.4	Defense response to Gram-negative bacteria	
Dro (*Drosocin*, *CG10816*)	↑ 3.4	Defense response to Gram-negative and Gram-positive bacteria	
AttC (*Attacin-C, CG4740*)	↑ 2.7	Defense response, antibacterial humoral response	
AttA (*Attacin-A*, *CG10146*)	↑ 2.3	Defense response to Gram-negative bacteria	
Mtk (*Metchnikowin*, *CG8175*)	↑ 2.5	Defense response to Gram-positive bacteria and fungi	
PGRP SB1 (peptidoglycan recognition protein-like, *CG9681*)	↑ 2.5	Defense response receptor activity, plays a role in the detection of bacteria	Only by Imd pathway (De Gregorio et al., 2002)
IM1 (Immune induced molecule 1, *CG18108*)	↑ 2.1	Defense response	-

Table 7 Immune response related genes whose transcript levels depend on the presence of SP in the abdomen of females, 4 h after copulation. Gene expression in females mated with SP^0 males was assumed as a background control value. “Fold change” was calculated for the expression level of the same gene in females mated with $Control^{SP}$ males related to the control value of the gene. In the column “Fold change”, the level of a gene expression in the females mated with $Control^{SP}$ males is compared with the level in females mated with SP^0 males. The arrow shows either an up-regulation (↑) or a down-regulation (↓) of the gene by SP.

Affymetrix ID	FlyBase ID	Fold change	Additional information
1. Unknown genes			
144998_at	*CG15745* (FBgn0030469)	↑ 2.6	-
152679_at	*CG10550* (FBgn0039321)	↑ 2.56	-
151980_at	*CG5150* (FBgn0035620)	↑ 2.55	Encodes product with alkaline phosphatase activity putatively involved in mesoderm development.
151094_at	*CG3348* (FBgn0040609)	↑ 2.3	Encodes product containing chitin binding domain, tachycitin.
145890_at	*CG9498* (FBgn0031801)	↑ 2.1	-
150652_at	*CG14558* (FBgn0039416)	↑ 2	-
149371_at	*CG1077* (FBgn0037405)	↓ 0.47	Encodes product that belong to Kazal-type serine protease inhibitor family, ovomucoid/PCI-1 like inhibitors.
144469_i_at	*CG15575* (FBgn0029699)	↓ 0.44	-
2. Others			
142154_at	*CG3234* (*timeless*, *tim*)	↑ 2	Mating behavior, regulation of circadian sleep/wake cycle
143879_at	*Rab-protein 1* (*Rab1*, *CG3320*)	↑ 2	RHO small GTPase

Table 8 Unknown genes and other genes that are regulated by SP in the abdomen, 4 h after copulation. Gene expression in females mated with SP^0 males was assumed as a background control value. "Fold change" was calculated for the expression level of the same gene in females mated with $Control^{SP}$ males related to the control value of the gene. In the column "Fold change", the level of a gene expression in the females mated with $Control^{SP}$ males is compared with the level in females mated with SP^0 males. The arrow shows either an up-regulation (↑) or a down-regulation (↓) of the gene by SP.

QRT-PCR verification of the microarray data

While microarray is a large scale technique which helps to find a large range of genes involved in establishment of the phenotype of interest, QRT-PCR is the method of choice to check a few genes of concern in a very accurate way. A QRT-PCR analysis was carried out to confirm the SP-dependent expression of the following genes observed by the microarray analysis: some of the antimicrobial peptide coding genes (*AttA*, *Dpt*, *DptB* and *Mtk*), a gene coding for a peptidoglycan-recognition protein (*PGRP-SB1*), and *timeless* (*tim*). The level of RNA expression for the mentioned genes was measured in the abdomen of females mated with SP^0 or $Control^{SP}$ males, respectively, (4 h after mating) by performing QRT-PCR. This analysis allowed obtaining more precise data concerning the fold change of the expression pattern of those genes (Fig. 17, Table 9). The level of gene expression in different conditions was calculated based on three different internal controls (actin, tubulin and RpL32). The level of expression of these housekeeping genes in the abdomen of females mated with SP^0 males was set to 100% or 1 (Fig. 17). A comparison of the microarray and the QRT-PCR data shows that they are consistent (Table 9). All genes coding for antimicrobial peptides and one PGRP and *tim* are up-regulated by SP in the abdomen of the females. The slight difference in the fold change obtained for these genes from microarray and QRT-PCR is due to the difference in the sensitivity of these approaches.

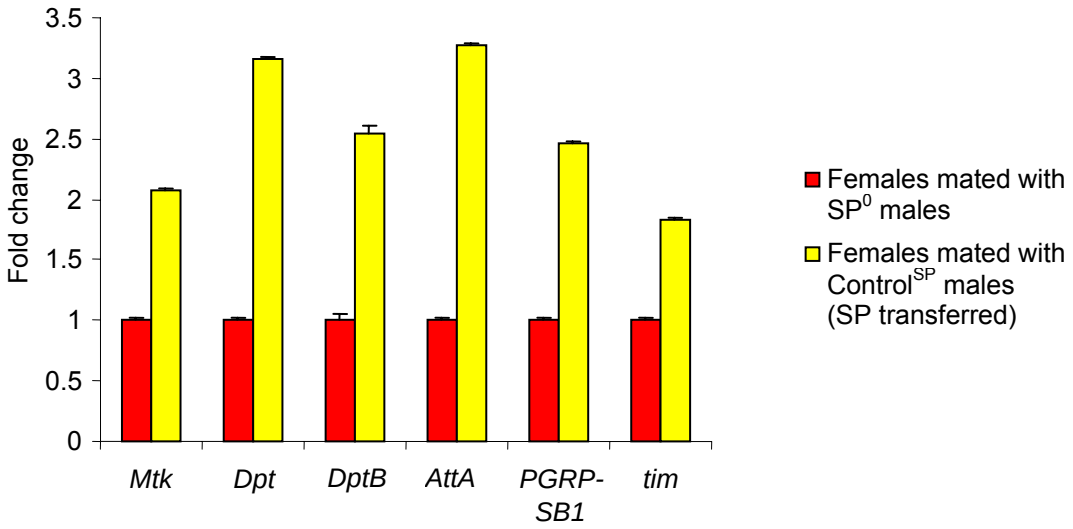

Fig. 17 QRT-PCR data of expression values for *AttA*, *Dpt*, *DptB*, *Mtk*, *PGRP-SB1* and *tim* in the abdomen of females mated with SP^0 males (in red) and females mated with $Control^{SP}$ males transferring SP (in yellow), 4 h after mating. Value of expression in the females mated with SP^0 males is set to 1. Standard deviations are indicated.

	QRT-PCR act	QRT-PCR tub	QRT-PCR RpL32	QRT-PCR mean	Microarrays
AttA	3.2	2.8	3.26	3	2.3
Dpt	2.7	2.4	2.8	2.65	3.4
DptB	3	2.65	3	2.9	3.7
Mtk	1.6	1.4	1.6	1.55	2.5
PGRP-SB1	2.3	2	2.35	2.2	2.5
tim	1.8	1.75	1.94	1.83	2

Table 9 Fold change of the expression levels of *AttA*, *Dpt*, *DptB*, *Mtk* and *PGRP-SB1* in the abdomen of females mated with Control^{SP} males in comparison to one in the abdomen of females mated with SP^{0} males (4 h after mating, based on the QRT-PCR data). The fold change was calculated according several internal normalization controls (actin, tubulin and RpL32), and the final result was calculated as a mean value.

3.2.1.3 Contribution of the C- and N-terminal parts of SP in the post-mating responses

The SP can be divided in two functionally independent elements: the N-terminal part (from aa 1 to 7) and the C-terminal part (from aa 8 to 36). While the N-terminal part is responsible for sperm binding (Peng et al., 2005a) and activation of juvenile hormone synthesis (Moshitzky et al., 1996), the C-terminal part of SP elicits the two PMR (Ding et al., 2003; Schmidt et al., 1993a). To determine which genes are regulated by the N-terminal part of SP and which ones by the C-terminal part of SP, the SP$^{\Delta 2\text{-}7}$ construct in a SP^{0} background (Peng et al., 2005a) was used for the following experiment. A microarray analysis was performed with RNA extracted from heads and abdomen of females mated with SP$^{\Delta 2\text{-}7}$ and Control $^{\Delta 2\text{-}7}$ males, respectively (4 h after copulation). According to the analysis of the microarray data there are no genes that show an expression pattern dependent on the N-terminal part of SP. This means that the expression of all genes described above (see 3.2.1.1 and 3.2.1.2) is controlled by the C-terminus of SP and is not dependent on the first 7 amino acids.

3.2.2 SP and the induction of the immune response

SP stimulates the innate immune response in the abdomen of mated females, but not in the head and thorax

According to the microarray data, 4 h after copulation the innate immune response is activated by SP in the abdomen of mated females (Table 7). Surprisingly none of the AMP genes controlled by SP in the abdomen showed any difference in the expression pattern in dependence of SP in the heads of mated females. To confirm this observation and to check the expression of the AMP genes in the thorax of females mated with SP^0 and $Control^{SP}$ males 4 h after copulation, a QRT-PCR analysis was performed for *Mtk*, *Dpt* and *AttA* genes. Wt females were mated with SP^0 and $Control^{SP}$ males, and 4 h after copulation RNA was extracted from heads and thoraces of mated females for further analysis. The level of gene expression in different conditions was calculated based on three different internal controls (actin, tubulin and RpL32). The expression of these genes was set to 100% in the head or thorax of females mated with SP^0 males (control values). The fold change of the expression level of genes in the head or thorax, respectively, of females mated with $Control^{SP}$ males was calculated in relation to the control values. The results show that, they are neither induced by SP in the heads nor in the thoraces of mated females (data not shown), although there is a clear up-regulation of AMP genes by SP in the abdomen of mated females (Fig. 17). Moreover, according the QRT-PCR data, the expression of the AMP genes is highly variable from experiment to experiment in the head and thorax of females, whereas it is tightly controlled in the abdomen (data not shown).

Analysis of expression of AMP genes reporter lines

To study the tissue specificity of the induction of the immune response by SP, the following available reporter lines, where marker genes (*GFP* or *lac*Z) are fused to the AMP gene promoters (Mtk-GFP and Drc-*lac*Z), were tested.

The Mtk-GFP reporter line contains 1.5 kb of *Metchnikowin* upstream sequence fused to the green fluorescent protein (GFP) cDNA and 790 bp of 3' flanking region of the *Drosomycin* gene, used to signal transcription termination and polyadenylation (Levashina et al., 1998). Unlike the product of the *Metchnikowin* gene, which is targeted by a signal sequence for secretion in the hemolymph, the GFP expressed from this construct remains within the cells where it is synthesized. In previous work (Levashina et al., 1998), GFP was induced upon immune challenge in larvae, and the kinetics of the protein expression was examined by Western blotting. Levashina et al. (1998) described the following important findings. The RNA transcripts could be detected

three hours after stimulation, and reached a plateau after 14 h, whereas the protein started to be detectable six hours after stimulation, and accumulate until 72 h before leveling off. This accumulation was proposed to reflect a high stability of GFP. The kinetics of the fluorescent level reflects the kinetics of GFP accumulation (Levashina et al., 1998). To check the tissue specificity of *Mtk* induction by SP, 5 d old females of the described transgenic line were mated with SP^0 and $Control^{SP}$ males, respectively, and also virgins were analyzed. Peng et al. (unpublished) have shown that the expression level of antimicrobial peptides is elevated after eclosion. Since GFP levels off 72 h after stimulation (Levashina et al., 1998), 5 d old females were used in the experiment to avoid the background signal of *Mtk* expression induced after eclosion. Females mated with SP^0 and $Control^{SP}$ males, respectively, were checked 6 h (Fig. 18) and 9 h after mating (data not shown). There was no difference observed in the GFP levels in females 6 or 9 h after mating.

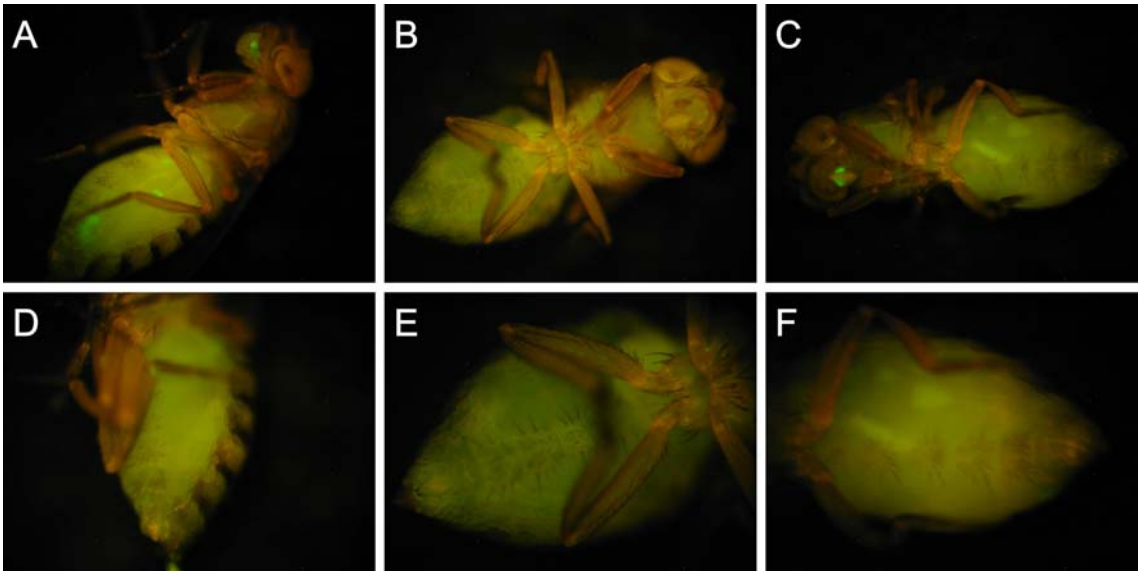

Fig. 18 GFP expression in the Mtk-GFP reporter line: virgin females (**A**, **D**); females mated with SP^0 males (**B**, **E**), 6 h after mating; females mated with $Control^{SP}$ males (**C**, **F**), 6 h after mating; whole flies (**A**, **B**, **C**); abdomen of analyzed females (**D**, **E**, **F**). The microscopic analysis was performed with a GFP2 filter.

It was impossible to discriminate the GFP expression levels between these three groups of females (Fig. 18). Already virgin females showed high levels of GFP expression. The reason could be that the difference between virgins and mated females is very small, and/or the microscopic analysis is not an enough sensitive approach for fly analysis in comparison to larvae.

Drc2.5-*lacZ*-2 is a reporter transposon consisting of a 2.5-kb *Drocosin* promoter fragment fused to the leader sequence of the bacterial *lacZ* gene, followed by *Drosocin* downstream sequences (Charlet et al., 1996). *A* constitutive expression of the Drc2.5-*lacZ*-2 reporter gene was observed

in the genital tract of female adults (Charlet et al., 1996). The expression was restricted to the calyx and oviducts of fertilized egg-laying females (Fig. 19, A) and was undetectable in virgin females (Charlet et al., 1996). To check whether this effect is due to SP transferred during mating, the Drc2.5-*lacZ*-2 reporter line females were mated with SP^0 males and with $Control^{SP}$ males. One, 2, 3, 4 and 6 h after copulation the β-galactosidase assay was performed for ovaries dissected from the mated females (Fig. 19). A strong endogenous β-galactosidase activity was observed in the uterus of Drc2.5-*lacZ*-2 reporter gene virgin and mated females, and also in wt females. A slight staining of the calyx, lateral and common oviducts of females mated with $Control^{SP}$ males was observed 1 - 2 h after mating but for none of the later time points. No similar staining was detected in virgin females and females mated with SP^0 males. Thus, the observed staining in females mated with $Control^{SP}$ males indicates that SP plays a role in the local regulation of the *Drosocin* expression in the female body. But a more precise analysis is needed to answer the question whether this staining appears in the genital tract of females or in the surrounding fat body cells.

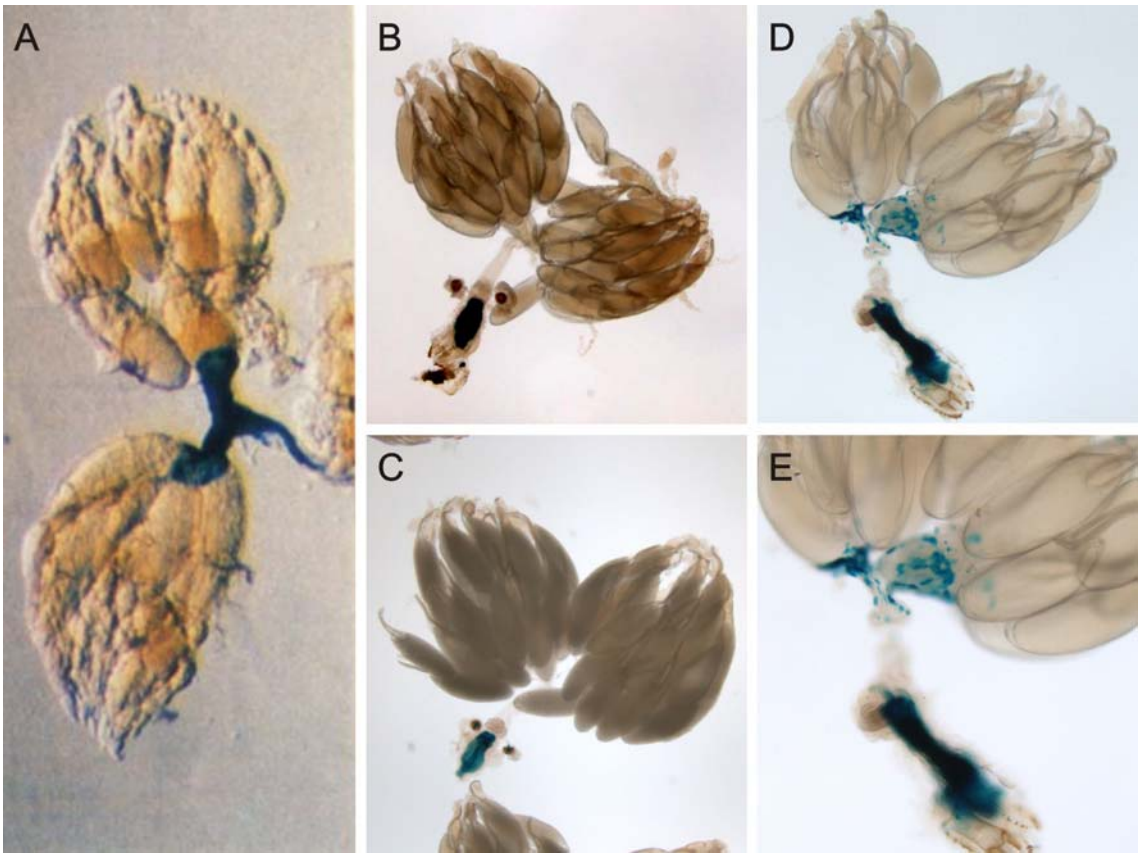

Fig. 19 Histochemical staining of β-galactosidase in Drc2.5-*lacZ*-2 transformants. Original picture (**A**, Charlet et al., 1996), ovary dissected from an unchallenged Drc2.5-*lacZ*-2 egg-laying female. The calyx, lateral and common oviducts are stained. Drc2.5-*lacZ*-2 virgin females (**B**); Drc2.5-*lacZ*-2 females mated with SP^0 males (**C**), 1 h after mating, no staining in the lateral and common oviducts is observed; Drc2.5-*lacZ*-2 females mated with $Control^{SP}$ males, 1 h after mating, slight dot staining in the lateral and common oviducts is observed (**D**, **E**). **E** is a higher magnification of **D**. The uterus and spermatheca staining is normal for wt females and due to the endogenous β-galactosidase activity.

3.2.3 Which part of SP elicits the immune response?

To elucidate the mechanism of the SP-dependent induction of the immune response, an analysis of the contribution of different fragments of SP to the stimulation of the innate immune response in the abdomen of females is necessary. According to the microarray analysis (3.2.1.3) performed with females mated to $SP^{\Delta 2-7}$ and $Control^{\Delta 2-7}$ males, there are no genes that are dependent on the N-terminal part of SP. That leads to the conclusion that the N-terminal part of SP (from the 1st to the 7th aa) is also not essential for the stimulation of the immune response (Fig. 20). To investigate which part of SP is important for the activation of the defense reaction in females, injection experiments with different fragments of SP, or S2 cell culture experiments with cells exposed to the different fragments of SP, may be performed.

Fig. 20 Amino acid sequence of SP and function of different fragments of SP. According to the microarray data (3.2.1.3), the N terminal part of SP (grey aa) has no contribution in the SP-dependent gene regulation 4 h after mating. Prolines marked in red are hydroxylated, non-modified prolines are underlined.

Injection of SP in to virgin females and the immune response

The aim of the injection experiment was to find out which specific part of SP is essential for eliciting the immune response in the abdomen of females. The injection of SP was performed into the thoraces (a widely used approach to study pathogen infection) and into the abdomen (a way to inject SP to study the two PMR) of virgin 5 d old females (Fig. 21, A and B). Four hours after injection, the abdomen of the females were cut off, RNA extracted, and QRT-PCR was performed for *AttA* (Fig. 21, A) and *Dpt* (Fig. 21, B), which have shown a SP dependent expression pattern in the abdomen of mated females (Table 7). As a control for fly handling, untreated, but cooled virgin females (Fig. 21, red) were used. Also, it was interesting to compare the level of AMP gene activation under native conditions (by mating), with the level elicited by injection. The flies mated with SP^0 males (Fig. 21, orange) and with $Control^{SP}$ males (Fig. 21, yellow) were analyzed. The

AttA and *Dpt* expression values for abdomen of females mated with SP^0 and $Control^{SP}$ males were included in the analysis (Fig. 21, A and B). To distinguish between the effects of injuring the fly per se and the SP-dependent immune stimulation, abdomen of females were pinched in the abdomen (Fig. 21, grey), or flies were injected with water either in the abdomen (Fig. 21, light green) or in the thoraces (Fig. 21, light blue). These controls were used to evaluate the background elevation of AMP gene expression resulting from the injury. The experimental flies were treated in the same way as the previously described controls, but in this case 50 μl solution containing 3pmol of SP were injected per female. According to the injection data, even water injection in the thorax or in the abdomen stimulates the immune response in the abdomen of females at a very high level. Hence, it is not possible to determine the effect of SP in the stimulation of the innate immune system by injection. Although injection experiments are a widely used approach in studying the immune response induced by pathogens, in the case of SP it is not suitable.

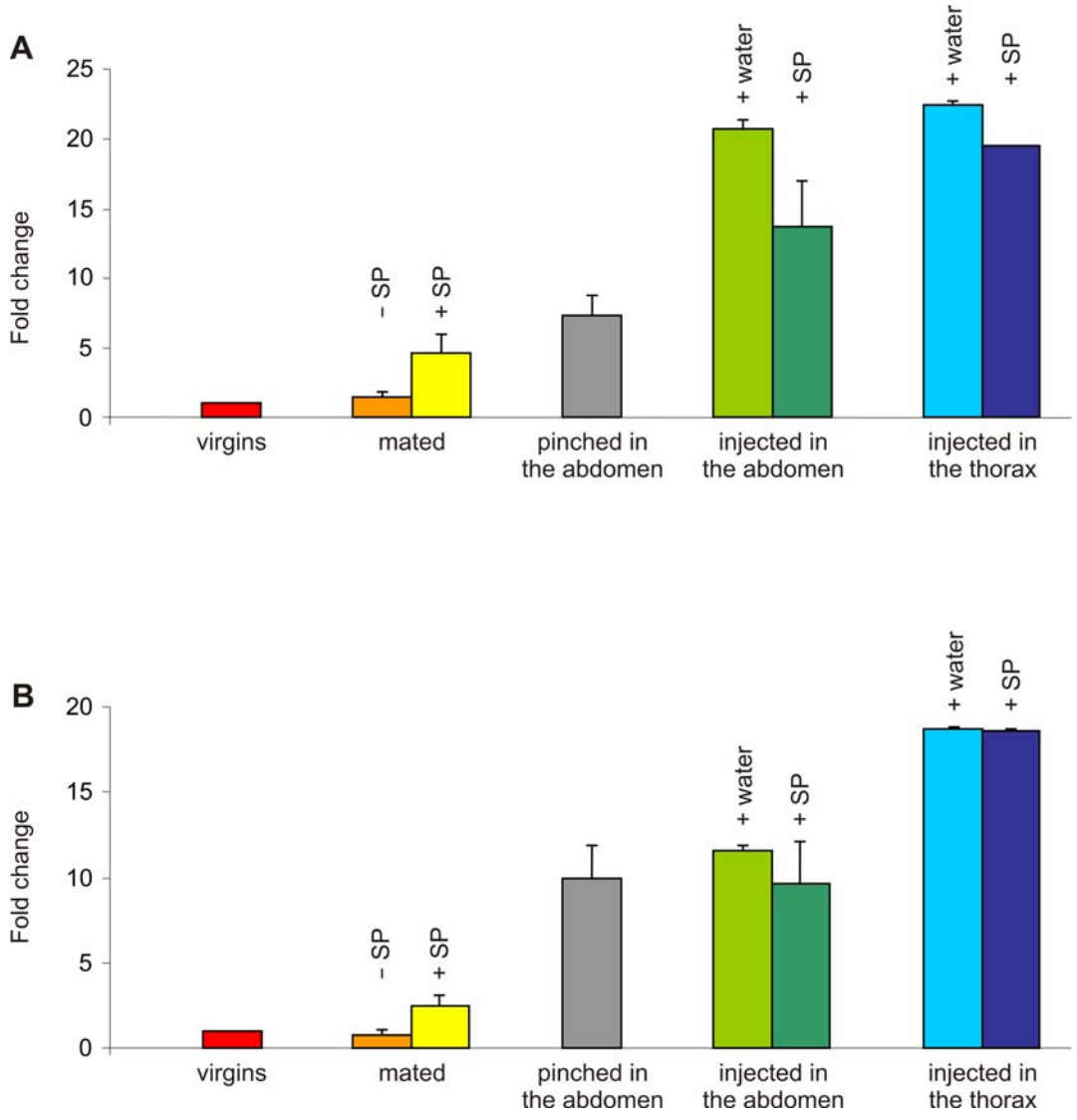

Fig. 21 Expression values of *AttA* (**A**) and *Dpt* (**B**) in the abdomen of differently treated females based on the QRT-PCR data. Virgin females, red; females mated with SP^0 males, orange, or with $Control^{SP}$ males, yellow; females pinched in the abdomen with a fine sterile needle, grey. Injection experiments: females injected in the abdomen with 25 μl of water, light green, or with 50 μl of 3 pmol SP, dark green; females injected in the thorax with 50 μl of water, light blue, or with 50 μl of 3 pmol SP, dark blue.

4 Discussion

Insects dominate terrestrial ecosystems, a success which is due to their physiology, high reproductive potential and enormous variety in reproductive strategies. Nevertheless, there are some common features which one can observe in a range of insects. Among these are the Accessory gland proteins (Acps) which are produced in males and transferred during mating into females. Acps play a major role in the modulation of female physiology and behavior after mating, and, thus, control the number and the genetic diversity of their offspring. Sex-peptide (SP), one of the Acps, is a key regulator of the two most conspicuous post mating responses (PMR): an increased oviposition, and decreased receptivity to courting males after mating. Although SP is a well studied molecule, the receptor of SP and most components of the SP response cascade are still unknown. In this study, based on the previous knowledge about the nature of the SP receptor and SP binding sites in the female body, I tried to identify and characterize the receptor of SP and components of the SP response cascade.

4.1 Search for the receptor of SP

The identification and characterization of the SP receptor at the top of the cascade is crucial to understand the mode of action of SP in the female body. Since it is not known to which class of receptors it may belong, a variety of different approaches was attempted. Thus, a cDNA expression library screen was carried out in COS cells with an alkaline phosphatase – SP fusion protein as a probe (Ding, 2002). The screen did not give any positive result, most probably due to the fact that it was not an optimal approach to fish out transmembrane proteins (Ding, 2002). Therefore, a homology approach was applied to find candidate genes for the role of the SP receptor. Indeed, two genes, *AICR2* and *star1*, were identified (Ding, 2002). Here, I checked if one of these genes is indeed a SP receptor. Transgenic flies carrying RNAi constructs for these genes (B. Dickson, IMBA, Vienna) were analyzed for the PMR. The results showed that both of these genes are involved in the establishment of the egg laying machinery, but do not affect the rejection behavior of females after mating. Hence, *AICR2* and *star1* are not implicated in the control of both PMR, i.e. none of them is the SP receptor (this conclusion holds under the assumption that there exists a single molecular receptor for both PMR). As a next step, I performed a microarray analysis based on the fact of the developmental regulation of the SP receptor (Ding et al., 2003). This approach was combined with a proteomics assay. Unfortunately, the only candidate gene, *GRHR*, identified by this experiment was not confirmed as a SP receptor by an analysis of GRHR transposon insertions.

Therefore, a new screen with the "DUALmembrane system" assay was carried out. The advantage of this system is that ligand – receptor interactions can be studied in the membrane, thus, under natural conditions. Indeed an interaction between SP and Off-track (OTK) was detected. A pairwise interaction assay needs to be done to confirm the binding of SP with OTK. OTK is a glycoprotein of molecular weight 160 kDa. The extracellular domain of OTK has six immunoglobulin (Ig) repeats and shows similarity to cell adhesion proteins (Pulido et al., 1992). OTK can mediate hemophilic adhesion, which results in tyrosine phosphorylation of the intracellular domain (Pulido et al., 1992). OTK itself seems not to be an active tyrosine kinase. Most probably, it belongs to a family of kinase "dead" receptors. The catalytic domain of OTK is altered in a few key conserved residues that are implicated in autophosphorylation (Winberg et al., 2001). OTK plays a role as a component of Plexin signaling during axon guidance (Winberg et al., 2001). It was proposed that the activated Plexin/OTK complex recruits the Rho exchange factor, providing local activation of Rho (Winberg et al., 2001). Unfortunately, our knowledge about OTK function in adult flies is elusive. Recent studies suggested a new role for OTK in the *Drosophila* odor memory (Buffone et al., 2004). The neuronal component of the SP response cascade (Ding et al., 2003; Ottiger et al., 2000), and the possible implication of OTK in memory formation in adult flies, support the suggestion that OTK could indeed be a receptor for SP. Contrary to this assumption is the difference in the temporal expression of OTK and the SP receptor. The SP receptor does not show a detectable expression in the CNS during the pupal stage and right after eclosion of the fly (Ding et al., 2003). OTK demonstrates another expression pattern in time. It is expressed throughout all developmental stages of the fly. OTK is broadly distributed in early *Drosophila* embryos. In later stages (larvae and adults), the protein is always detected on neuronal cell bodies and axons within the CNS and in the projection of motor neurons as they extend to the muscle fibers in the periphery (Pulido et al., 1992). This means that OTK is constantly expressed during the changes of the spatial expression pattern.

The OTK loss-of-function mutants are lethal. Thus, to check whether OTK is the SP receptor, the following experiment was done. In most cases, reducing the gene dose of two co-interacting proteins may generate a mutant phenotype. This effect was demonstrated for the OTK/Plexin interaction (Winberg et al., 2001). Since, the SP receptor demonstrated a SP dose-dependent manner of activation (Schmidt et al., 1993a), it was hypothesized that if one copy of the wild type *otk* gene is present in females, and if OTK is a receptor for SP, these flies will not respond to the injection of the minimal working concentration of SP. Hence, otk^{3} -/+ females (Winberg et al., 2001) were injected with different concentrations of SP. However, these flies did not show any difference in SP sensitivity in comparison with wt females, which indicates that there is no interaction between SP and OTK. But in view of the fact that in the case of a SP/receptor

interaction, the manner of binding and activation of the downstream components is not clear, I can not conclude that OTK is not the SP receptor. I have to remind that the experiment done above was based on the assumption that the SP/OTK interaction works in the same manner as the Plexin/OTK complex.

Outlook: future perspectives to check whether OTK is the SP receptor

In sum, a new promising candidate gene was found. To confirm this finding, however further experimental work has to be done e.g. the pairwise interaction of SP with OTK *in vitro* (will be done by "DUALmembrane system", Zurich). Another possibility is to test directly if OTK responds electrophysiologically to SP, which would be further evidence for a biologically significant interaction (will be done by G. Carvalho, Benzer Lab, California Institute of Technology). To confirm *in vivo* that OTK is the SP receptor, a conditional knock out with RNAi could be done.

4.2 Downstream components of the SP response cascade

My aim was to elucidate the components of the SP response cascade leading finally to the two PMR in the female. There are two molecularly different SP binding proteins in the female body: one localized in the nervous system, another one in the genital tract (Ding et al., 2003). An interesting question was which genes are changing their expression pattern due to the presence of SP in the head and in the abdomen of a female, respectively. The RNA transcript levels for the whole *Drosophila* genome were characterized for wt females (heads and abdomen separately) mated with males of different genotypes. Experimental females were mated either with SP^0 males or with $Control^{SP}$ males which allowed to identify the genes controlled by SP. I took 4 h after mating as a time point for the analysis to detect the earliest genes involved in the SP response cascade. Also, by analyzing females mated with $SP^{\Delta 2\text{-}7}$ males, I could answer the question of the contribution of the N- (from aa 2 to 7) and the C-terminal (from aa 8 to 36) parts of SP in gene regulation. Thus, I showed that all genes identified by the microarray experiment are controlled by the C-terminal part of SP from aa 8 to 36, and the N-terminal part is not contributing to their regulation. This indicates that the N-terminal part of SP does not have any additional function besides the stimulation of juvenile hormone synthesis (Moshitzky et al., 1996) and sperm binding (Peng et al., 2005a). This finding is consistent with data showing that the C-terminal part of SP from aa 8 to 36 is essential and sufficient for the control of the two PMR (Ding et al., 2003). Besides this, a new function, stimulation of the immune response, was described for the C-terminal part of SP (see 3.2.2 and 4.2.3.2).

4.2.1 SP elicits differential gene expression in the head and abdomen of females

There are two target sites for SP in the *Drosophila* female body: one in the nervous system (CNS and PNS), the other in the genital tract (Ding et al., 2003; Ottiger et al., 2000). There are two molecularly different binding proteins present at these sites. Neuronal binding is more demanding in terms of SP aa sequence, while genital binding is less specific. To find out which genes are controlled by SP in the CNS and which in the genital tract, respectively, the SP-dependent gene expression was examined by microarray analysis in the head and in the abdomen separately. Among the genes displaying changes in mRNA expression, those which demonstrated a more than 2 fold change were selected. This resulted in a set of 34 SP-regulated genes in the heads of mated females and 18 in the abdomen. According to the microarray data, SP does not change the expression pattern of its target genes more than 6 fold and the majority of changes are in the range of 2-3 fold change. This finding indicates that while the presence of SP leads to crucial changes in the behavior and physiology in the female, the peptide has a moderate effect on gene expression.

An interesting observation was that none of the SP-controlled genes in the head were present in the set of SP-dependent abdominal genes. This observation leads to the conclusion that SP elicits differential gene expression in the abdomen and in the heads of females. This may be due to the presence of an independent receptor for SP in the genital tract of the female which may lead to the activation of a different signaling cascade. So far, it was assumed that a transporter in the genital tract of the female helps SP to enter the hemolymph, whereas a receptor in the CNS, at the top of SP response cascade, leads to the PMR. Based on the finding of the differential gene expression induced by SP in these two parts of the body, the old hypothesis may have to be revised. The binding protein of the genital tract may play an independent role in the modulation of the female physiology after mating. Alternatively, it could be a SP receptor and transporter at the same time.

4.2.2 Target genes of SP in the head

Four hours after mating, 34 genes showed a SP-dependent expression pattern in the heads of females. Among the genes with a described function, the majority is represented by genes involved in metabolism (8 genes, Table 3) and signal transduction (7 genes Table 5). Also, 4 genes coding for proteases whose transcript levels depend on the presence of SP were found (Table 4). A further detailed analysis of mutants is needed to understand how all these genes are involved in the support of the PMR. The analysis of genes involved in signal transduction and

their involvement in the establishment of the two PMR might help to understand the nature of the SP signaling cascades. Since we do not know enough to speculate too much about all these components, I would like to concentrate on the changes in metabolism and proteolysis induced by the presence of SP.

The egg production machinery is stimulated by SP, and the females enormously increase their egg laying rate. Since the process of egg production is energy and resource consuming, it is not surprising that the food preferences (G. Carvalho, personal communication) and the metabolism in general change in females after mating. These results were confirmed by a proteomics approach (Huanfa Liu, personal communication). Metabolic enzymes were also found by a 2-D gel differential analysis of proteins extracted from heads or abdomen + thoraces of females mated with SP^0 and $Control^{SP}$ males, respectively. Since 24 h was taken as a time point in the proteomics approach, it is not possible to compare directly the list of metabolic genes from the microarray experiment with the list of metabolic enzymes found by proteomics. They are not overlapping in the single elements, but the main tendency of changing the energy/nutrition mode in the female is consistent.

Another interesting group of SP-controlled genes is a group of genes encoding proteases (Table 4). Two trypsins (λ and γ), one serine protease, and one serpin (serine protease inhibitor) are down-regulated due to the presence of SP. Recently, it was shown in some other studies that trypsins, serine proteases, and serpins are influenced by mating (Lawniczak and Begun, 2004; McGraw et al., 2004). This finding is very interesting in terms of male – female molecular interactions and sexual conflict. It was described before that serine proteases and serpins are present in the seminal fluid (Coleman et al., 1995; Swanson et al., 2001). Male-derived serpins are involved in the impediment of female proteases of modifying Acps (Wolfner, 2002). On the other hand, it is very common that Acps are cleaved by proteases when they are transferred to the females. This cleavage quite often requires proteolytic components from both sexes (Park and Wolfner, 1995). Thus, the presence of proteases and serpins in the seminal fluid of males and their involvement in the post-mating response of females is very interesting in terms of sexual conflict and the evolution of manipulative and resistance traits of males and females (Arnqvist and Rowe, 2005; Eberhard, 1996).

In view of the facts mentioned above, what is the functional meaning of the down-regulation of protease encoding genes by SP? One of the possible explanations is the following. By inhibiting proteolytic activities of cells, the half-lives of the peptides which are activated by SP and necessary for the two post-mating responses are prolonged. This might result in a prolonged SP action and, thus, represent an increase of male competitiveness. On the other hand, the

processing of some Acps which might be necessary for the establishment of full PMR could be inhibited and, as a result, a female could increase the number of her mating partners. Since the down-regulation of proteolytic components is observed in the heads of the females and not in the abdomen, the first suggestion makes more sense. Also, there are other potential functions for the inhibition of the genes coding for proteases: an implication of the products of those genes in the regulation of neuropeptides (Nijhout, 1994) or in the immune response (Table 6, Table 7). Trypsin-like serine proteases and their inhibitors, serpins, have been shown to play a central role in the insect immune response. For instance, the activation of the Toll ligand Spaetzle is under the control of a still unknown serine protease cascade and at least one serpin, Necrotic (Levashina et al., 1999). Hence, to come to a conclusion about the actual function of the down-regulation of these proteolytic components, more data about the functional biology of these molecules are necessary.

Among SP-dependent genes with unknown functions found in the head, 4 encode products with predicted tachycitin domains (Table 6). As tachycitin is an antimicrobial protein with chitin-binding activity in horseshoe crab hemocytes (Kawabata et al., 1996), these genes could be involved in the microbial defense in *Drosophila*. There are some known *Drosophila* proteins containing a chitin-binding type-2 domain that is involved in the immune response. For example Hemolectin, which is expressed in embryonic and larval hemocytes (Goto et al., 2003). The antimicrobial function and the mode of action of the tachycitin domain against bacteria and fungi are unclear. So far, it is unknown how tachycitin could be connected to the described innate immune response mechanism, the Toll and the Imd pathways.

4.2.3 Target genes of SP in the abdomen

Among the 18 SP-regulated genes in the abdomen, half of them are genes with unknown function (Table 8). Furthermore only two of them are down-regulated by SP (belonging to the group of genes with unknown function). The other 16 genes are up-regulated due to the presence of SP. In the group of genes with known function, the majority is coding for innate immune response peptides (7 AMPs and one peptidoglycan recognition protein, PGRP-SB1, see Table 7), and only one of them is encoding for a circadian clock protein, timeless (tim).

4.2.3.1 SP-dependent induction of *timeless* in the abdomen

One of the most intriguing results from the microarray experiments was the up-regulation of *timeless* (*tim*) expression by SP in the abdomen of mated females. The gene *tim* is a circadian

clock gene, which is ubiquitously expressed in the nervous system and in the peripheral tissues. While clock genes in the brain are essential for behavioral rhythms, the physiological role of these genes in the periphery are not well understood (Beaver et al., 2003). Recently, it was discovered that wt *D. melanogaster* displays a circadian rhythm of mating activity that is controlled by the circadian clock genes *tim* and *period* (*per*), and that the females are responsible for generating the mating rhythms (Sakai and Ishida, 2001). Since mating activities of the per^{01} and tim^{01} mutants were elevated in the morning (Sakai and Ishida, 2001), the up-regulation of *tim* by SP might play an inhibitory role in the female mating activities. Surprisingly, *tim* showed control of its expression by SP in the abdomen of mated female and not in the heads. This leads to a contradiction with the fact that the female needs the optic lobe, including the lateral neurons, to generate these mating rhythms (Sakai and Ishida, 2001). Based on these data, I suggested, that the SP-dependent regulation of *tim* might have another function, and that is most probably not involved in the inhibition of the female mating rhythm.

Recently, *timeless* (*tim*) and *period* (*per*) were shown to be involved in a novel noncircadian function in the ovary. PER and TIM are constantly expressed in the follicle cells that envelop young oocytes at the previtellogenic and early vitellogenic stages (Beaver et al., 2003). PER and TIM play a significant role in the reproductive fitness of *D. melanogaster*: Per^{01} and tim^{01} flies were shown to lay a lower number of eggs per couple, and it was proposed that the clock mutants might compensate for their lower reproductive fitness by re-mating more often (Beaver et al., 2002). Thus, based on the described role of *tim* for reproductive fitness and the proposed higher rate of re-mating of tim^{01} females, I proposed the following model (Fig. 22, A). Since *tim* seems to be involved in the regulation of both egg laying and re-mating, in other words, it affects both PMR resulting from SP action, I proposed that *tim* might be involved in the SP response cascade before it is separated into two independent branches. Thus, *tim* might be one of the earliest downstream components of the SP response cascade.

To test the hypothesis, the PMR of mated tim^{01} females were checked more precisely. Although, the oviposition of mated tim^{01} females was indeed decreased (Fig. 16), the rejection behavior was the same as for wt mated females (data not shown). Thus, *tim* can not be involved in the SP response cascade at such an early stage as proposed. The gene *tim* might be involved in this cascade already after it is branched into two independent responses, and it belongs to the branch which controls egg laying rate (Fig. 22, B). The genes *per* and *tim* affect female fertility via their role in the process of oogenesis (Beaver et al., 2003). However, the mechanism of the noncircadian regulation of *tim* and *per* in oogenesis is still unknown. But taken together, these data and the fact that *tim* has a SP-dependent expression profile, suggest that SP might be involved in this novel noncircadian mechanism which affects oogenesis

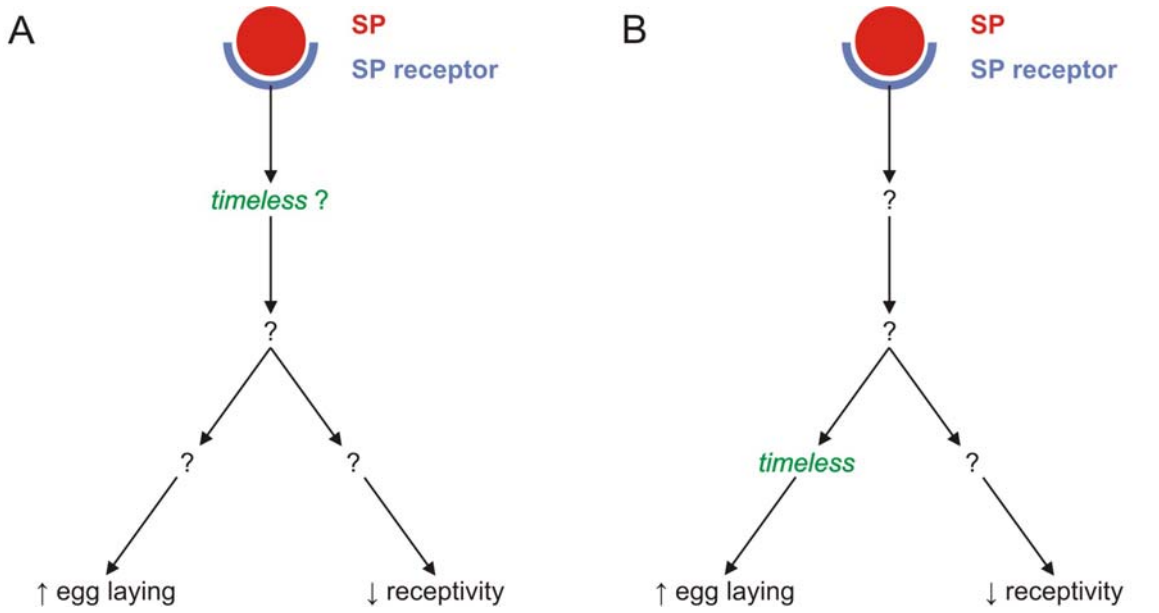

Fig. 22 The possible involvement of *timeless* in the SP response cascades. **A** *tim* is hypothesized to be involved in the SP cascade before it is separated in two independent branches, and, thus, contributes to the control of both PMR: egg lying and receptivity. **B** *tim* is placed in the SP cascade after it is separated in two independent branches, and, thus, contributes only to the egg lying control and does not influence receptivity of the fly.

4.2.3.2 SP and the immune response

Activation of the immune response in females after mating was already described in recent studies (Lawniczak and Begun, 2004; McGraw et al., 2004). In this work, SP was shown to be the key regulator of the immune response stimulation. Peng et al. (2005b) performed a detailed QRT-PCR analysis for some immune response genes in virgin and mated flies of different genotypes, and these QRT-PCR data are in agreement with the microarray findings. It was discovered that mating, due to the presence of SP, rapidly and transiently stimulates the transcription of AMP genes (Peng et al., 2005b). In mated females *Mtk*, *Drs* and *Dpt* induction was observed already 1 h after mating. The peak of activation is reached 2 – 4 h after mating, and within 8 h transcription drops to the virgin level (Peng et al., 2005b). As the *Mtk* expression is always on the same high level in Yp-SP females (transgenic females expressing SP ectopically and constitutively, Aigaki et al., 1991), it was proposed that the level of AMP gene expression is dependent on the SP concentration. Since the uterus of a female is enriched with SP right after mating followed by a decrease in SP concentration after 1 h (Matthias Soller, personal communication), it is not surprising that the immune response is only transiently induced by SP. Furthermore, the induction of the immune response by SP in the abdomen (3.2.2), but not in the rest of the female body, might be due to the difference in the concentrations of SP there. Below, I discuss two main

questions concerning the SP-dependent induction of the immune response in the female. What is the molecular mechanism of the SP-dependent stimulation of the immune response? What is the biological meaning of this induction?

The mechanism of SP action in the control of the immune response

So far, the insect immune system was thought to depend only on a small number of molecules that are recognizing common features of microbes, the so called innate immune response. The innate immune response is activated by receptors that recognize surface determinants that are conserved among microbes, but absent in the host, such as lipopolysacharides, peptidoglycans and mannans (Medzhitov and Janeway, 1997). Flies respond to a microbial challenge by the activation of proteolytic cascades leading to coagulation and melanisation of the hemolymph, the phagocytosis and encapsulation of invading microorganisms by blood cells, and the secretion into the hemolymph of combinations of antimicrobial peptides (AMPs) (Hoffmann and Reichhart, 2002). There are two main pathways which control the transcription of AMP encoding genes: the Toll and the Imd pathways. AMPs are secreted from the immune organs, mainly the fat body, which is a functional analogue of the mammalian liver. To date, seven distinct families of AMPs were identified in *Drosophila*: Cecropins (Kylsten et al., 1990), Diptericins (Wicker et al., 1990), Drosocins (Bulet et al., 1993), Attacins (Asling et al., 1995), and the insect Defensins (Dimarcq et al., 1994) are selectively active against bacteria, whereas Drosomycins are only active against fungi (Fehlbaum et al., 1994). The seventh group, Metchnikowin, is unique among the antimicrobial peptides of *Drosophila* since it is active against both bacteria and fungi (Levashina et al., 1995). Recently, evidence for an unsuspected molecular complexity of the innate immune system of insects was found (Watson et al., 2005). The gene called *Dscam* (Down syndrome cell adhesion molecule) is expressed in more than 18,000 isoforms in immune-competent cells of *Drosophila*. It is proposed that all these isoforms of Dscam function like primitive antibodies recognizing specific pathogens. It is not clear yet which mechanism controls the adaptive Dscam transcription in response to pathogens.

Based on the microarray experiment, SP induces a range of AMP genes which are known to be unspecifically induced as a result of several different infections (Roxstrom-Lindquist et al., 2004) by either the Toll or the Imd pathways But SP does not control *Dscam* expression. Dscam is predicted to have a high recognition specificity since the different versions of Dscam bind with different affinities to the bacterium *E. coli* (Watson et al., 2005). Hence, we can propose that the immune response activated by SP is not orientated towards any precise infection, and most probably boosts the immune response as a preventive mechanism. The genetic dissection of the Toll and the Imd pathways showed that SP affects the Toll pathway at or upstream of the gene

spaetzle, and the Imd pathway at or upstream of the gene *imd* (Peng et al., 2005b). But to understand how SP can activate the Toll and the Imd pathways a further detailed analysis is needed.

Understanding which part of SP is responsible for the stimulation of AMP gene transcription may help to comprehend the mode of SP action on the innate immune system. So far, two functional parts of SP were distinguished: the hydrophobic N-terminal end (from aa 1 to 7) responsible for sperm binding and stimulation of the JH synthesis, and the hydrophilic C-terminal end (from aa 8 to 36) eliciting the two PMR (Fig. 23). In this study, a new function was found for SP – the stimulation of the immune response in female abdomen. As described above (3.2.1.3), the microarray experiments with transcripts isolated form heads and abdomen of females mated with Control$^{\Delta 2\text{-}7}$ or with SP$^{\Delta 2\text{-}7}$ males revealed that the N-terminal end does not contribute to the gene regulation by SP 4 h after mating. Thus, the innate immune response is regulated by the C-terminal part of SP from aa 8 to 36. The C-terminal end can be divided into two parts. One, from aa 8 to 21 is a unique part for SP, the other, from aa 22 to 36, is conserved within the SP family and has a high homology to the very C-terminal part of DUP99B (Fig. 1). This part includes the disulfide bridge. Thus, if the C-terminal part of SP (from aa 22 to 36) would be responsible for the innate immune stimulation, then DUP99B should be able to induce AMP gene expression at least at some level as well. When females are mated to SP^{0} males, they still receive DUP99B, and thus they should demonstrate elevated levels of AMP gene expression in comparison to virgin females. In fact, this is not the case, since according to the QRT-PCR data the level of immune response gene transcripts in virgin females is statistically not different form the one in females mated with SP^{0} males (Fig. 21, A and B). This finding indicates that DUP99B does not contribute, or only marginally, to the innate immune response stimulation, which leads to the conclusion that the part of SP from aa 22 to 36 does very likely not elicit AMP gene expression. Thus, the middle part of SP (from aa 8 to 21) is most probably the functional part in the control of immunity (Fig. 23). A very particular feature of this part of SP is the special pattern which is formed by prolines, P-X-X-X-P-X-P-X-P-X-P. Furthermore, all these 5 prolines are hydroxylated. The function of this posttranslational modification, so far, was not clear. Additionally, two non-hydroxylated prolines are located in the N-terminal and very C-terminal end of SP. Combining all these facts, we suggest the following working hypothesis. The hydroxylation of the prolines and the particular structure formed by the pattern of hydroxyprolines are essential for the immune function of SP (Fig. 23).

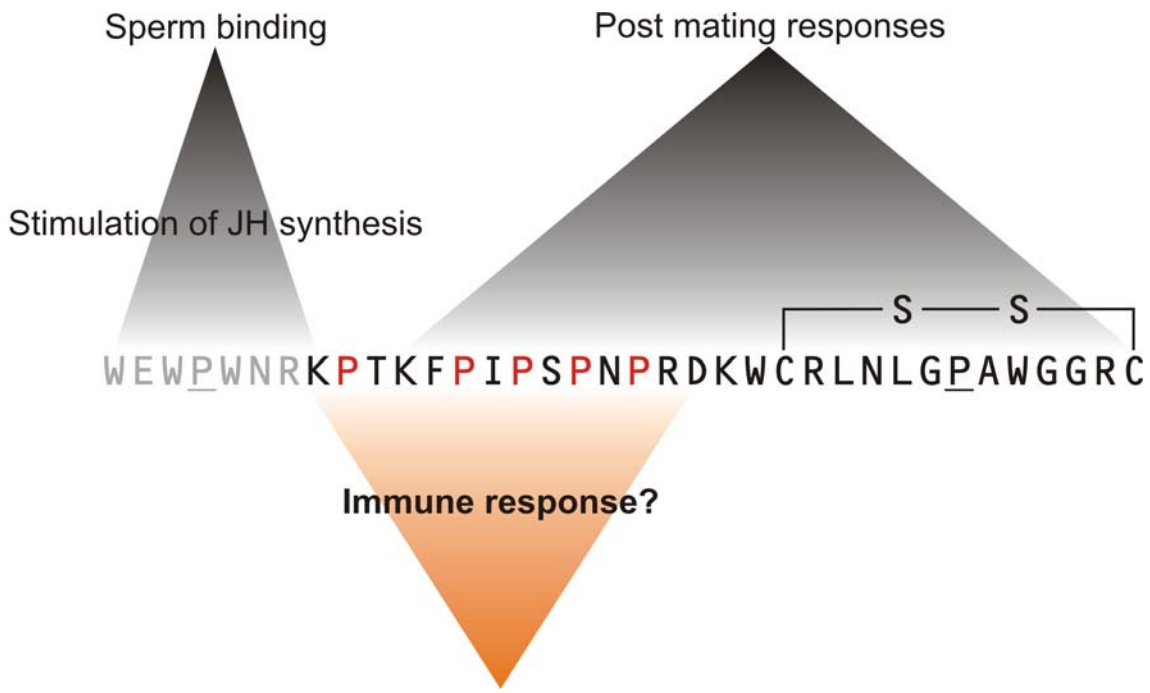

Fig. 23 Structure – function relationships of SP. Results from *in vitro* and *in vivo* experiments. According to the microarray data (3.2.1.3), the N terminal part of SP (marked in grey) makes no contribution in the gene regulation 4 h after mating. It is hypothesized that the middle part of SP is responsible for the immune response activation. Prolines marked in red are hydroxylated; non-modified prolines are underlined.

One possible mode of action could be “mimicry”. The middle part of SP containing the 5 hydroxyprolines may be able to mimic peptidoglycans exposed on the surface of pathogens. Hence, it may be recognized as a pathogen-specific determinant, and, thus, induce the two immune response pathways.

One possibility to confirm the hypothesis would have been to inject different synthetic fragments of SP with and without posttranslational modifications into females, and to subsequently monitor the immune response. Unfortunately, the injection experiments are not suitable for this purpose. The QRT-PCR data clearly show that the induction of the immune response by a control injection is much higher than after mating. Thus, it was not possible to determine the contribution of SP in the injection experiments (Fig. 21). One way to avoid the problem of injuring the fly would be to produce transgenic males with a SP^0 background expressing only the middle part of SP containing different modifications. However, this assay is very time consuming. Furthermore, the short form of SP could be modified differently *in vivo* than the full length peptide. An alternative is to carry out the experiment *in vitro*, in *Drosophila* S2 cells. S2 cells are macrophage-like cells that are intensively used as a model system for studying immunity (Ramet et al., 2002). S2 cells incubated with lipopolysaccharides (LPS) rapidly mount the immune response (Foley and O'Farrell, 2004). Hence, it should be possible to incubate S2 cells with SP to induce the AMP

gene expression. Different fragments of synthetic SP with and without hydroxylation could be added to the medium of S2 cells and 2-4 h after incubation the level of the immune response gene expression could be measured.

The biological meaning of SP-dependent induction of the immune response

The last point which I would like to discuss concerning SP and the immune response induction is the biological meaning of this event. One possible explanation is the preventive function of the SP-dependent induction of the immune response acting as defense of the female. Mating is a risky business for the female since physical damage during copulation is frequent which may expose the female to pathogens transferred by the male. Hence, SP could rapidly boost the immune response during the first hours after copulation to prepare the female body for a possible pathogenie attack. On the other hand, it is known that the costs of activation of the immune response leads to the reduction of some other fitness traits, for example fecundity (Fellowes et al., 1999). Thus, males may not be necessarily interested in defending females if this leads to the reduction of the progeny number. Hence, the defensive role of the induction of the AMP gene transcription could be orientated not to the female but to the progeny. Males transfer during mating some protective peptides (Wolfner, 2002). For example Acp62F, a trypsin inhibitor, is found on the surface of the first eggs laid by mated females, where it might exert a protective function (Lung et al., 2001). Thus, AMPs produced under the control of SP in females could be deposited on the first egg laid by females and play the same function as Acp62F. The spatial analysis of the SP-dependent immune response showed that it occurs most probably locally in the genital tract. The analysis of the Drc2.5-*lacZ*-2 reporter line females indicated that β-galactosidase staining occurred in the lateral oviducts of females mated with Control^{SP} males (Fig. 19). Thus, the hypothesis about the protective role of the immune response for the progeny is in agreement with the latter results. The eggs are moving towards the uterus and on the way they could be covered by a range of the AMPs synthesized/secreted into the lateral oviduct. The second hypothesis is in accord with the interest of the male to find a balance between the energetic and the nutritional costs of the immune response activation and high fecundity.

Outlook: future perspectives to study SP-dependent immune response activation

The SP-dependent induction of the immune response in the females is a very interesting observation in terms of mechanism of the induction, evolutionary perspectives in terms of sexual conflict, and also the biological meaning of this event. To determine the mechanism of SP action on the immune response, the analysis of a range of mutants producing truncated or modified versions of SP is needed. Also, as mentioned above, the experiment with S2 cells incubated with

different fragments of synthetic SP should be continued. To check the biological meaning of the immune response induction, experiments with infected of females after mating with SP^0 and $Control^{SP}$ males, and a further comparative analysis of these flies and their progeny to survive could be done. Recently, natural pathogens were described for *D. melanogaster* (Roxstrom-Lindquist et al., 2004; Vodovar et al., 2005), which could be used for our purposes.

5 Appendix

The DUALmembrane system (MbYTH)

The yeast two-hybrid system plays an essential role in detecting or confirming protein-protein interactions among nuclear and cytoplasmic proteins. This approach allows to identify novel interaction partners and provides hints to their function. However, membrane proteins, such as receptor tyrosine kinases, G protein–coupled receptors, membrane-bound phosphatases, and transporters are difficult to study using classical protein interaction assays because of their hydrophobic nature. Recently, a new system was described which allows the identification of integral membrane-interacting proteins (Iyer et al., 2005; Stagljar et al., 1998). This so-called "split-ubiquitin membrane-based yeast two-hybrid assay" (MbYTH or DUALmembrane system) involves fusing the halves of ubiquitin to two interacting proteins, at least one of which is membrane bound. Upon interaction of these two proteins, the halves of ubiquitin are brought together, and the transcription factor that is fused to a membrane protein of interest is cleaved and released. The free transcription factor then enters the nucleus and activates transcription of reporter genes.

DUALmembrane technology

The MbYTH was designed to overcome the limitation of the conventional YTH, which is based on reconstitution of transcription factor (TF) activity that must occur in the nucleus. The MbYTH is based on the observation that ubiquitin can be experimentally separated into two moieties that functionally reconstitute when present in close proximity to one another (Johnsson and Varshavsky, 1994). Ubiquitin is a small, evolutionarily conserved protein, which when covalently attached to a target protein marks that protein for degradation by the 26*S* proteosome. Whereas the target protein degradated by the 26S proteosome, the ubiquitin motifs are saved from degradation by the ubiquitin-specific proteases (UBPs). UBPs cleave the ubiquitin from the target protein and hence recycle the ubiquitin back to the cytoplasm (Glickman and Ciechanover, 2002). In the MbYTH, a membrane protein of interest, "bait," is fused to the C-terminal half of ubiquitin (Cub), along with an artificial TF that consists of the bacterial LexA-DNA binding domain and the *Herpes simplex* VP16 transactivator protein. The putative interacting proteins, "prey," are either membrane-bound or cytosolic, and are fused to the N-terminal half of ubiquitin (Nub). Because of their high affinity, the two halves of ubiquitin spontaneously reconstitute and are recognized by the UBPs. Hence, to prevent spontaneous association, an isoleucine (I) to glycine (G) exchange at position 13 of the Nub moiety has been introduced (NubG) (Johnsson and Varshavsky, 1994).

Only upon interaction of the two proteins (prey and bait) does the reconstitution of ubiquitin (Cub + NubG) occur; this so-called split ubiquitin is then recognized by abundant UBPs, resulting in the cleavage of the TF. The released TF then enters the nucleus and activates the transcription of the reporter genes, which then can be monitored by growth on selective plates or by colorimetric assays. The reporter strains used in this system have the *HIS3*, *lacZ*, and/or *ADE2* reporter genes (Fig. 24). The detailed protocol is described in (Iyer et al., 2005).

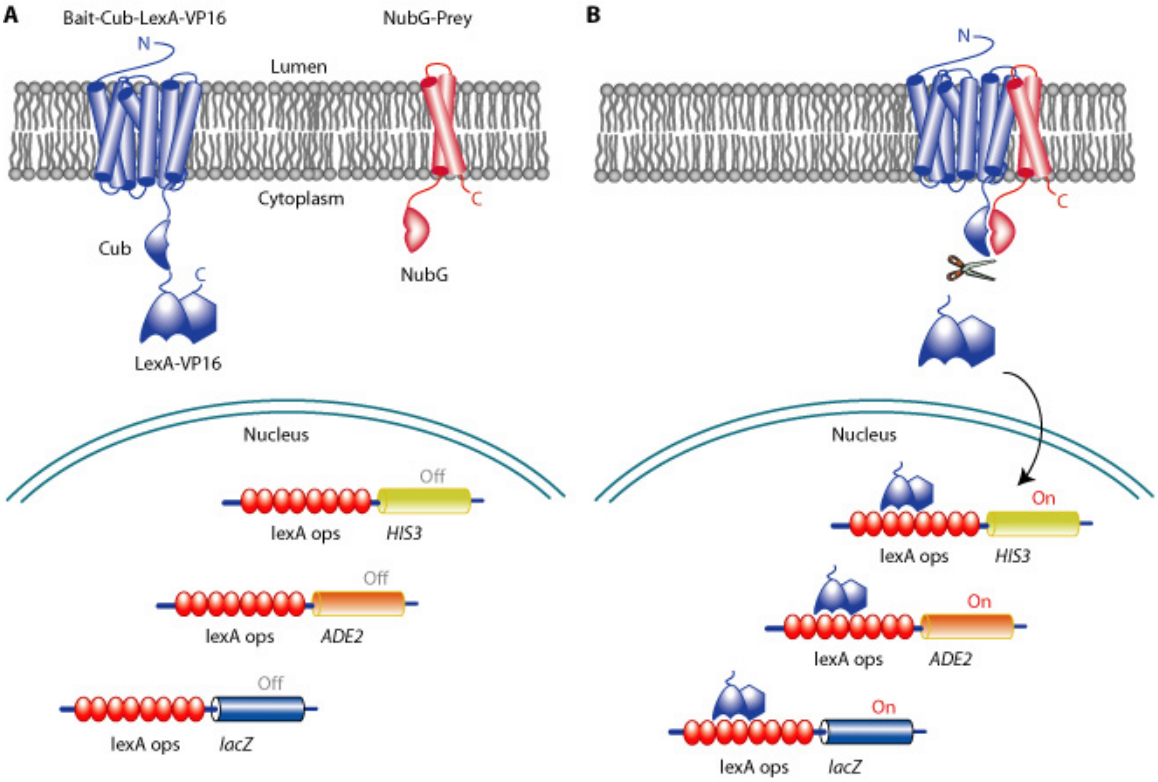

Fig. 24 Outline of the membrane YTH system. (A) A membrane bait protein of interest is fused to Cub followed by the artificial transcription factor LexA-VP16 (blue), while another prey membrane (or cytoplasmic) protein is fused to the NubG domain (red). If the bait and the prey do not interact, there is no reconstitution of ubiquitin and no UBP-mediated cleavage of the transcription factor occurs; this results in HIS3−/ADE2− and *LacZ*− yeast. (B) On interaction of the bait and prey proteins, ubiquitin reconstitution occurs, leading to proteolytic cleavage by UBPs and the subsequent release of the transcription factor. This factor enters the nucleus and activates reporter genes by binding to the Lex A operator sites (lexA ops) within their promoters. This results in HIS3+/ADE2+ and *lacZ*+ yeast cells (in case of a three-reporter strain, for example, THY.AP4) (from Iyer et al., 2005).

DUALmembrane baits

In the MbYTH, a given transmembrane bait can either be a Type I (C-terminus in the cytosol) or Type II (C-terminus outside the cell or in the lumen of ER, Golgi etc.) transmembrane (TM) protein. In the case of a Type I TM protein, the Cub-TF portion is fused to its C-terminus so that the Cub-TF portion faces the cytosol, thus generating a Bait-Cub-TF fusion. If a bait to be studied in the MbYTH is a Type II TM protein, the TF-Cub portion has to be fused to N-terminus (TF-Cub-Bait). In both cases, an interaction between an integral membrane protein and a prey protein will result in the cleavage after the last amino acid of the Cub domain, thus liberating either TF (in the case of the Type I TM bait) or TF-Cub (in the case of Type II TM bait) (Fig. 25).

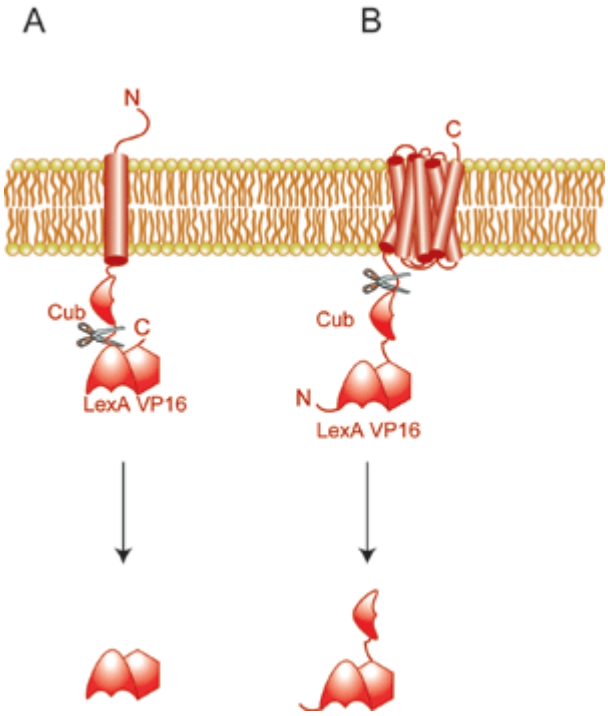

Fig. 25 Schematic representation of the type I transmembrane bait (A) and type II transmembrane bait (B) (from www.dualsystems.com).

DUALmembrane preys

In the Dual membrane system, a given prey protein (or a cDNA library) can be studied in either a Y-NubG (where Y is cDNA or genomic DNA insert) or NubG-Y orientation. In the case of a Type I TM prey, the cDNA (or a library of cDNAs encoding potential interactors) is fused at its C-terminus with the NubG domain, whereas in the case of a Type II prey, it is fused at its N-terminus with the NubG domain. In this way, it is possible to identify both Type I (Y-NubG orientation) and Type II (NubG-Y orientation) transmembrane proteins that interact with a particular membrane bait protein (Fig. 26).

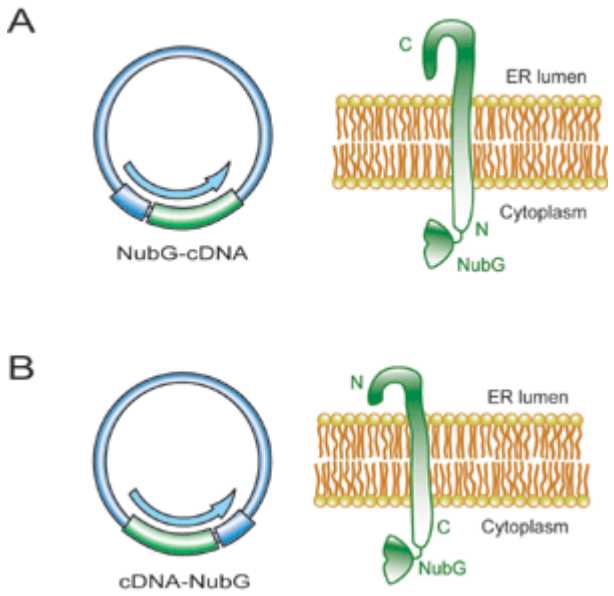

Fig. 26 Schematic representation of the type I transmembrane prey (A) and type II transmembrane prey (B) (from www.dualsystems.com).

One of the advantages of MbYTH system is that it can be used to monitor the interaction either between two membrane proteins or between a membrane and a cytosolic protein.

Proof of concept

So far, several laboratories have successfully applied the MbYTH system using proteins from a wide range of organisms. For instance, the system was applied to isolate interacting partners of the human proteins, BAP31 nad ErbB3, from human cDNA libraries (Thaminy et al., 2003). Furthermore, the MbYTH system was also used to study the interaction between plant sucrose transporters (Reinders et al., 2002), FE65 protein and its implication in APP binding and regulation of secretion (Sabo et al., 1999).

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CURRICULUM VITAE

Surname: DOMANITSKAYA

First name: Elena

Date of birth: January 29, 1979

Place of birth: Novosibirsk, Russia

Nationality: Russian

Educational history:

1994 – 1996
High school, Novosibirsk, Russia

1996 – 2001
Novosibirsk State University, Department of Biological Sciences, Novosibirsk, Russia
M.Sc. diploma thesis title: "Peculiarity of polytenization of precentric heterochromatin regions of the chromosome 3 of *Drosophila melanogaster*".

2001 – 2002
Research student in the laboratory of Molecular Cytogenetics supervised by
Prof. I. F. Zhimulev, Institute of Cytology and Genetics, Novosibirsk, Russia

2002 – 2005
PhD thesis in the laboratory of Reproductive Behavior of Drosophila supervised by Prof. Dr. E. Kubli, Institute of Zoology, University of Zurich, Switzerland
PhD thesis title: “Search for the receptor and target genes of Sex-peptide”

Publications / abstracts:

I. Primary

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